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Targeting Calcium Sensitization Mechanisms In Human Airway Smooth Muscle For Novel Bronchodilator Therapy In Asthma

Edwin Yoo
University of Pennsylvania, edwinjyoo@gmail.com

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Abstract
Asthma is characterized by airway hyperresponsiveness (AHR) and inflammation. Management of asthma involves controlling airway inflammation with inhaled corticosteroids, and using bronchodilators such as β-2 agonists that inhibit airway smooth muscle (ASM) shortening and prevents or reverses airway narrowing. Despite β-2 agonists' ability to mitigate asthma exacerbations, they have limitations, especially concerning efficacy and safety. Studies have shown that β-2 agonist use can result in adverse patient outcomes, β-2 adrenergic receptor tachyphylaxis, deterioration of asthma control, and death. New therapeutics are needed to overcome these limitations. Rho kinase (ROCK) inhibitors have generated excitement as potential bronchodilators. Since ROCK activation is necessary to maintain ASM tone by inhibiting MLC phosphatase, ROCK inhibitors allow the constitutively active MLC phosphatase to de-phosphorylate MLC and promote relaxation of ASM. ROCK inhibitors blunt ASM contraction and induce bronchodilation in vitro, ex vivo, and in vivo. Unfortunately, ROCK is ubiquitously expressed and adverse effects such as vasodilation preclude any clinical trials. We present an alternate strategy to induce bronchodilation in human airways by inhibiting the p110δ isoform of phosphoinositide 3-kinase (PI3Kδ) that is not ubiquitously expressed. In primary human ASM cells (HASMCs), we demonstrate that PI3Kδ modulates ROCK activation, and that inhibition of PI3Kδ using a FDA approved drug Cal101 (Idelalisib) attenuates ROCK activation. Cal101 induces relaxation of cultured HASMCs by inhibiting PI3Kδ-mediated activation of ROCK. Using human precision-cut lung slices, we also demonstrate that Cal101 induces bronchodilation of human small airways as effectively as formoterol, an industry standard β-2 agonist. In our model of β-2 agonist tachyphylaxis, the effectiveness of β-2 agonists was significantly attenuated while the effectiveness of Cal101 was not. Thus, our results suggest that PI3Kδ inhibitors represent a novel class of bronchodilators. Furthermore, we demonstrate that Gα12 mediates agonist-induced activation of PI3K and ROCK. These results unveil a novel pathway regulating HASMC signaling that is necessary to elicit shortening. Importantly, our work has uncovered several new targets for bronchodilation and offers new avenues for the treatment of asthma.

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Reynold A. Panettieri

Second Advisor
Benjamin A. Garcia

TARGETING CALCIUM SENSITIZATION MECHANISMS IN HUMAN AIRWAY SMOOTH MUSCLE FOR NOVEL BRONCHODILATOR THERAPY IN ASTHMA

Edwin J. Yoo

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Supervisor of Dissertation

_____

Reynold A. Panettieri, Jr.

Professor and Vice Chancellor for

Translational Medicine and Science

Co-Supervisor of Dissertation

_____

Benjamin A. Garcia

Presidential Professor of

Biochemistry and Biophysics

Graduate Group Chairperson

_____

Julie A. Blendy, Professor of Pharmacology

Dissertation Committee

Hydar Ali, Professor, Professor of Pathology

Lawrence F. Brass, Professor of Medicine and Pharmacology

Blanca E. Himes, Assistant Professor of Informatics

Kelly L. Jordan-Sciutto, Chair and Professor of Pathology

ABSTRACT

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Dr. Reynold A. Panettieri, Jr.

Asthma is characterized by airway hyperresponsiveness (AHR) and inflammation. Management of asthma involves controlling airway inflammation with inhaled corticosteroids, and using bronchodilators such as β-2 agonists that inhibit airway smooth muscle (ASM) shortening and prevents or reverses airway narrowing. Despite β-2 agonists' ability to mitigate asthma exacerbations, they have limitations, especially concerning efficacy and safety. Studies have shown that β-2 agonist use can result in adverse patient outcomes, β-2 adrenergic receptor tachyphylaxis, deterioration of asthma control, and death. New therapeutics are needed to overcome these limitations. Rho kinase (ROCK) inhibitors have generated excitement as potential bronchodilators. Since ROCK activation is necessary to maintain ASM tone by inhibiting MLC phosphatase, ROCK inhibitors allow the constitutively active MLC phosphatase to de-phosphorylate MLC and promote relaxation of ASM. ROCK inhibitors blunt ASM contraction and induce bronchodilation *in vitro*, *ex vivo, and in vivo*. Unfortunately, ROCK is ubiquitously expressed and adverse effects such as vasodilation preclude any clinical trials. We present an alternate strategy to induce bronchodilation in human airways by inhibiting the p110δ isoform of phosphoinositide 3-kinase (PI3Kδ) that is not ubiquitously expressed. In primary human ASM cells (HASMCs), we demonstrate that

PI3Kδ modulates ROCK activation, and that inhibition of PI3Kδ using a FDA approved drug Cal101 (Idelalisib) attenuates ROCK activation. Cal101 induces relaxation of cultured HASMCs by inhibiting PI3Kδ-mediated activation of ROCK. Using human precision-cut lung slices, we also demonstrate that Cal101 induces bronchodilation of human small airways as effectively as formoterol, an industry standard β-2 agonist. In our model of β-2 agonist tachyphylaxis, the effectiveness of β-2 agonists was significantly attenuated while the effectiveness of Cal101 was not. Thus, our results suggest that PI3Kδ inhibitors represent a novel class of bronchodilators. Furthermore, we demonstrate that $G\alpha_{12}$ mediates agonist-induced activation of PI3K and ROCK. These results unveil a novel pathway regulating HASMC signaling that is necessary to elicit shortening. Importantly, our work has uncovered several new targets for bronchodilation and offers new avenues for the treatment of asthma.

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CHAPTER 1: Introduction and Comprehensive Literature Review

Edwin J. Yoo

Rutgers Institute For Translational Medicine and Science, Child Health Institute, Rutgers University, New Brunswick, NJ

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Asthma and Airway Smooth Muscle

Asthma is characterized by airway hyperresponsiveness (AHR), inflammation, and reversible bronchoconstriction. It is a major contributor to morbidity, mortality, and healthcare expenditures in the United States, costing approximately $50 billion yearly in direct healthcare costs[1]. The mainstay of asthma treatment consists of corticosteroids to prevent inflammation and bronchodilators like β–agonists, which reverse and prevent bronchoconstriction by relaxing ASM. Unfortunately, about 50% of asthmatics have inadequate control with current therapeutics[2]. Evidence suggests that the regular use of β-agonists increases AHR[3,4], worsens asthma control[2,5], and increases morbidity and mortality[5–7]. Truly novel bronchodilators that overcome the limitations of current drugs have yet to emerge.

PI3K Activation and Function

Initially discovered in studies of carcinogenesis, PI3K mediates cell functions including proliferation, metabolism, and motility – all of which are important to cancer progression[8]. Other studies determined that PI3K plays a role in a myriad of diseases.

PI3K phosphorylates the D-3 position of the plasma membrane lipid phosphatidylinositol-4-5-bisphosphate [$PI(4,5)P_2$], generating phosphatidylinositol-3-4-5-trisphosphate [$PI(3,4,5)P_3$][9,10]. Cytosolic signaling proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3K activation by direct binding to $PI(3,4,5)P_3$. Proteins with PH domains include protein kinases, scaffolding proteins, and guanine exchange factors (GEFs), all of which will become activated and initiate signaling cascades[11–14]. Notable effectors of PI3K signaling include protein serine-threonine kinases Akt and phosphoinositide-dependent kinase 1 (PDK1), as shown in Figure 1. Binding of Akt and PDK1 to $PI(3,4,5)P_3$ brings these proteins into proximity, inducing

phosphorylation of Akt by PDK1. Activation of Akt by PDK1 activates mammalian target of rapamycin complex 1 (mTORC1) and a host of other proteins, affecting cell growth and proliferation[15].

Signaling of the PI3K pathway terminates by dephosphorylation of $PI(3,4,5)P_3$ by the enzyme phosphatase and tensin homolog (PTEN), the main endogenous PI3K inhibitor[16]. PTEN is a tumor suppressor and is important in many cancers. The PI3K pathway is also inactivated by Src-homology domain 2 (SH2)-containing phosphatases SHIP1 and SHIP2, found primarily in blood cells[17].

PI3Ks consist of heterodimers composed of a p110 catalytic subunit and a regulatory subunit. The PI3K family is divided into three classes (class I, II, and III) based on structural and functional characteristics, as shown in Figure 1. Further divided into subclasses based on their p110 catalytic subunit, class IA PI3Ks are composed of p110α, p110β, and p110δ isoforms while class IB contains solely the p110γ isoform. Class IA PI3Ks contain p85, p55, or p50 phosphotyrosine-binding regulatory subunits. Class IB PI3Ks contain p101 or p84/p87 regulatory subunits, that allow activation by the βγ subunits of G-protein coupled receptors (GPCRs)[18]. Class II PI3Ks consist of three isoforms (C2α, C2β, and C2γ) that are ubiquitously expressed[19,20]. Class III PI3K includes only one member, vacuolar protein sorting mutant 34 (Vps34p), that regulates endocytosis, toll-like receptor signaling, and vesicular trafficking[21]. Classes II and III are not well studied due to the lack of highly selective pharmacological inhibitors. Class I is the most thoroughly studied PI3K family, and will be the focus of this review.

Functions of Class I PI3K

Due to the limitations of selective inhibition strategies, the study of Class I PI3K family members remains challenging[22]. Unlike the p110δ and p110γ subunits, the p110α and p110β subunits are required for cell proliferation and embryonic development[23]. Consequently, *in vivo* experiments requiring genetic knockdown of p110α and p110β pose obstacles.

The p110δ and p110γ isoforms are predominantly, but not exclusively, expressed in leukocytes, and play important roles in innate and adaptive immune responses[24]. Activated toll-like-receptors (TLRs) in leukocytes recruit p110δ and p110γ and initiate signaling[25]. In T-cells, p110δ and p110γ negatively regulate TLR-induced interleukin-12 and interferon-γ production, facilitating Th1 responses and diminishing Th2 responses[26]. p110δ knockout mice and mice expressing inactive p110δ catalytic subunit demonstrate impaired CD28-costimulated clonal expansion and differentiation, highlighting the necessity of p110δ in T-cell activation[27,28]. Interestingly, patients with activated PI3K delta syndrome (APDS) manifest increased susceptibility to airway infections, bronchiectasis, and lymphoproliferation[29]. p110γ deficient mice display reduced thymocyte survival and T-cell maturation, with a significant decrease in $CD4^+$ cells[30]. PI3K also mediates B-cell function as p110δ knockout mice display impaired B-cell receptor-mediated antigen presentation[31]. In neutrophils, PI3K regulates NADPH Oxidase and is important for reactive oxygen species generation. Stimulation of numerous neutrophil receptors including GPCRs, cytokine receptors, integrin receptors, and Fc receptors, activates GEFs. GEFs, such as PRex1 and Cytohesin4, mediate chemotaxis, vesicle trafficking, degranulation, and NADPH Oxidase activation[32].

PI3K serves to promote immune cell survival by modulating anti-apoptosis signaling. The PI3K/AKT pathway inhibits pro-apoptotic proteins including B-cell lymphoma 2 (Bcl2) and associated proteins. PI3K signaling also facilitates activation of pro-survival proteins including BCL-XL, MCL-1, and NF-κB[33].

Collectively, these studies demonstrate that p110δ and p110γ are integral to the orchestration of both the innate and adaptive immune responses, including leukocyte migration, activation, B-cell and T-cell maturation, neutrophil NADPH Oxidase activation, and antigen response.

PI3K and Asthma

Atopic asthma manifests when T-cells mature into a Th2 subtype upon allergen exposure and release mediators that activate other immune cells such as mast cells, granulocytes, and B-cells. Activated immune cells then elicit responses from structural cells such as ASM and airway epithelial cells, which culminate in AHR, inflammation, and remodeling. PI3Ks play important roles in the responses of airway immune cells and structural cells that mediate these pathophysiological processes.

The importance of PI3K in asthma is demonstrated by *in vivo* experiments that show that PI3K inhibitors prevent pathogenesis of allergen-induced AHR and inflammation[34,35]. IC87114, a p110δ selective inhibitor, attenuated allergic airway inflammation and AHR in a murine model[36]. p110δ also mediates lung inflammation induced by Aspergillus fumigatus via a mechanism involving endoplasmic reticulum stress[37]. Additionally, allergen-induced AHR does not develop in p110γ-deficient mice[38]. Taken together, these experiments suggest that PI3K is necessary for the development of asthma.

PI3K and Asthma: Structural Cells

Structural cells, including airway smooth muscle cells and epithelial cells, are the main effector cells of inflammatory mediators released during asthma. Airway smooth muscle cells proliferate and shorten upon exposure to inflammatory mediators, inducing airway remodeling and obstruction[39]. Epithelial cells recruit eosinophils by releasing eotaxin. Eosinophils subsequently release major basic protein, inducing epithelial damage[40]. PI3Ks play an important role in mediating both ASM and epithelial cell responses.

ASM, the pivotal cell type mediating AHR, is the primary target for bronchodilation, a major therapeutic strategy. In asthma, ASM maintains airway tone, secretes inflammatory mediators, and undergoes hypertrophy and hyperplasia. ASM shortening occurs upon agonist binding to a GPCR, resulting in an elevation of intracellular calcium, myosin light chain (MLC) phosphorylation, and actin-myosin cross-bridge cycling, via the canonical inositol trisphosphate and calmodulin mediated pathway. In parallel, inhibition of myosin light-chain phosphatase by Rho Kinase (ROCK) sustains MLC phosphorylation and maintains ASM tone.

PI3K activation is necessary for the modulation of ASM contraction and the accumulation of contractile proteins[41,42]. Importantly, PI3K contributes to airway tone via its regulation of ROCK. In human *ex*-vivo small airways contracted to agonist, PI3K inhibitors evoke bronchodilation[43]. P110δ and p110γ subunits are required for the development of AHR in mice[36,44,45]. Furthermore, cytokine-mediated induction of CD38, a calcium signaling protein important to the development of AHR, was impaired following treatment with PI3K inhibitors[46]. P110γ activity was found to be elevated in ASM derived from subjects with asthma, and was also important for β-2 adrenergic

receptor resensitization[47]. These studies highlight the importance of PI3K in ASM contraction and AHR development.

ASM also secretes chemokines and cytokines such as IL-6, VEGF, and CXCL-8 that contribute to the recruitment of immune cells in asthma. PI3K is necessary for IL-6 secretion induced by TGFβ, a cytokine important to airway remodeling and AHR development[48]. Under mechanical strain, ASM signals through PI3K/Akt/mTOR and ERK pathways, inducing HIF-1α, a transcription factor required for VEGF expression[49]. VEGF release by mechanical strain of HASM may account for the angiogenesis seen after repeated asthma exacerbations[49]. Additionally, PI3K and MAPK pathways regulate the synergy of IL-17 and IL-1 beta to enhance CXCL-8 expression[50]. Taken together, these studies highlight the importance of PI3K to ASM chemokine and cytokine secretion.

In addition to ASM contraction and mediator secretion, PI3K plays an important role in airway remodeling. Airway remodeling refers to the structural changes that occur during asthma. ASM undergoes hyperplasia and hypertrophy to increase ASM mass. Th2 cytokines modulate airway contraction by secreting MMP-1 from the ASM cells via PI3K activation[51]. PI3K is also required for growth factor-induced cell migration[52–55]. Furthermore, activation of class IA PI3K is sufficient to stimulate DNA synthesis and growth, that promote airway remodeling[56].

ASM derived from subjects with asthma display increased proliferation, responsiveness to contractile agonist, and mediator release, suggesting an epigenetic alteration. Small RNAs are essential to the establishment of an epigenetic signature, and profiling of small RNAs suggests that the PI3K pathway is enhanced in bronchial smooth muscle cells from patients with asthma[57].

The airway epithelium, considered an essential modulator of inflammation, lie at the interface between the host and the environment. The epithelium represents the first

line of defense against microorganisms, toxicants, and allergens and express many pattern recognition receptors (PRRs) to rapidly detect and respond to pathogen-associated molecular patterns (PAMPs) found in microbes or to damage-associated molecular patterns (DAMPs) released upon tissue damage, cell death, or cellular stress. Activation of epithelial PRRs releases cytokines, chemokines, and antimicrobial peptides, that attracts and activates innate and adaptive immune cells. Studies show that endothelial cell activation is a key-triggering event in the recognition of inhaled allergens that activates the local network of dendritic cells (DCs), that coordinate the subsequent immune response. PI3K mediates epithelial responses to environmental stimuli. PI3K is important during viral-induced asthma exacerbations due to it's importance in virus internalization[58,59]. In an allergen-induced model of asthma, PI3K activation is increased and PTEN activation is decreased in the airway epithelium. PTEN protein expression and PTEN activity were also decreased in epithelial cells of mice exposed to allergen. Immunoreactive PTEN localized in epithelial layers around the bronchioles of control mice; PTEN rapidly disappeared in allergen-exposed lungs of mice, suggesting that the PI3K/PTEN pathway modulates epithelial cell function in asthma[60].

PI3K also facilitates mediator release from epithelial cells[61,62]. PI3K inhibition prevents expression of IP-10; a mediator released during virus-induced asthma exacerbations[63]. PI3K also modulates iNOS and NO signaling in the airway epithelium, promoting the development of airway inflammation[63,64]. P110δ inhibition attenuates antigen-induced airway inflammation and hyperresponsiveness through the modulation VEGF-induce vascular leakage[65]. PI3K inhibition also reduced the mucus hypersecretory phenotype and goblet cell metaplasia induced by IL-13, an important cytokine that is associated with asthma[66].

PI3K and Asthma: Immune Cells

Allergen exposure induces Th2 differentiation of T-cells that in turn secrete cytokines that promote allergic inflammation. Th2 cytokines stimulate B-cells to produce IgE and other antibodies. These cytokines include IL-4, that stimulates the production of IgE, IL-5, that activates locally recruited eosinophils, and IL-13, that stimulates mucus secretion from bronchial submucosal glands and promotes IgE production by B cells. As in other hypersensitivity reactions, IgE coats submucosal mast cells, and repeat exposure to the allergen triggers the mast cells to release granule contents and produce cytokines and other mediators, that collectively induce an asthma phenotype.

T-cells, in part, promote the development of asthma, secreting cytokines that are sufficient to induce AHR and B-cell class switching to IgE. Class I PI3Ks play roles in T-cell activation, differentiation, and proliferation, with p110δ and p110γ being the main contributors, as shown in Figure 2. Upon activation by antigen presenting cells, naïve CD4+ cells will proliferate and differentiate to various T helper effector subsets, including the Th2 subtype. Class I PI3Ks have important roles in T-cell functions. T-cell receptor engagement by antigen activates p110δ through tyrosine kinase signaling cascades[67]. p110γ is recruited by G-protein activation through GPCRs, that include chemokine receptors[68]. Mice lacking both p110δ and p110γ are markedly impaired in T-cell development, and are unable to rearrange the T-cell receptor α and β chains[69]. In naïve CD4+ T-cells, evidence suggests that class I PI3Ks are activated upon engagement by antigen presenting cells[70]. PI3Ks are required for the differentiation of Th effector subsets, with Th2 differentiation being necessary for the development of atopic asthma[28]. B-cells secrete IgE into circulation, that will then bind to high-affinity FcεR1 receptors on the surface of mast cells. PI3K negatively regulates IgE expression and IgE cell surface

receptor expression levels. Blockade of PI3K signaling markedly enhanced B-cell IgE class-switching and increased IgE levels in vivo, despite reduced type-2 cytokine production[71,72].

Mast cells, the central effector cell in allergic diseases, are present in increased numbers in the airways of patients with asthma. Binding of allergen to IgE on the cell surface induces a signal transduction cascade that releases mediators such as histamine and prostaglandin D2 (PGD2). The release of histamine and PGD2 evokes bronchoconstriction. IgE-mediated mast cell degranulation is augmented by PI3K[73]. Additionally, PI3K was found to mediate VEGF release in murine models, which in turn promotes vascular leakage in asthma[74].

Increased numbers of eosinophils are also present in the airways of some patients with asthma. The recruitment, growth, and survival of eosinophils is promoted by factors released from airway epithelial cells, Th2 cells, and mast cells. Eosinophils express a variety of proinflammatory cytokines, Th2 cytokines, and chemokines that can activate mast cells and stimulate the epithelium. Eosinophils can also present antigen to T cells, and release growth factors such as TGF-β, promoting inflammation in asthma. Inhibition of PI3K blunts eosinophil chemotaxis, a function important in asthma[75,76]. Additionally, PI3K induces mediator release in allergic asthma, irrespective of allergen challenge model[77]. Eosinophils derived from subjects with atopic asthma, have elevated PI3K activation, suggesting that PI3K may be an important mediator of eosinophil function in asthma[78].

Neutrophils are present in some, but not all, patients with asthma. Neutrophilic migration and activation releases mediators that contribute to asthma exacerbations. PI3K is necessary for neutrophil migration and degranulation in asthma [77,79].

PI3K Inhibition as a Potential Therapeutic Strategy for Asthma

PI3K has gathered much attention as a potential therapeutic target in asthma as outlined in Table 1[80]. Pan-inhibitors of class I PI3K (wortmannin and LY294002) have toxicity profiles and unfavorable pharmacokinetic characteristics, that preclude their clinical use[81]. The focus, therefore, has shifted to isoform-selective PI3K inhibitors. PI3Kα inhibitors have been studied in the treatment of solid tumors but have not been approved for clinical use as shown in Table 2 [82]. PI3Kδ and PI3Kγ, as well as dual inhibitors, are promising candidates. PI3Kγ inhibitors exhibit anti-inflammatory properties, but none have been approved for clinical use. Idelalisib, a PI3Kδ inhibitor, has been approved for the treatment of multiple hematologic malignancies. Side effects such as leukopenia, colitis, and skin rashes have occurred upon treatment with idelalisib[83]. Given the anti-inflammatory and bronchodilator effects, PI3Kδ inhibition may be a promising strategy for clinical development, especially with inhaled formulations to minimize side effects. Dual PI3Kδ/PI3Kγ inhibitors have also been effective in mitigating inflammatory disorders in animal models, and may be an alternate approach[81]. IPI-145 (duvelisib) and RV-1729 are PI3Kδ/PI3Kγ combination inhibitors that are being developed for clinical use[79,84]. TG100-115, another PI3Kδ/PI3Kγ combination inhibitor, has been effective in allergen-induced asthma models[85]. GSK2269557, an inhaled PI3Kδ inhibitor, is being developed for severe asthma[84].

AKT and mTORC1 are activated downstream of PI3K and oral inhibitors including miltefosine and rapamycin have been developed for clinical use. None of these drugs have been tested for use in airway diseases. Rapamycin has shown promise in

inhibiting mTOR activation in COPD[86] and inhibits allergic inflammation in allergen-challenged mice, while inhibiting eosinophil differentiation [87,88].

Although promising, PI3K inhibition as a therapeutic strategy is not without challenges. Problems include lack of specificity, adverse effects, and loss of efficacy. PI3K inhibitors like most kinase inhibitors target the ATP-binding pocket. Adequate inhibitor potency must be achieved in order to compete with ATP for binding. The structural similarity of the ATP-binding pocket in all kinases makes specific targeting difficult. Having an improved understanding of the ATP-binding pocket structure may target the development of inhibitors with improved specificity and potency. Adverse effects occur due to the myriad important functions of PI3K in other tissues. Inhaled delivery systems can promote topical deposition while minimizing system effects. Development of inhaled PI3K inhibitors with adequate potency has been difficult, but the many preclinical studies and early clinical trials provide hope for future success of PI3K inhibition in airway diseases.

Asthma and Bronchodilation

Asthma management has historically focused on: 1) using bronchodilators that bind G protein-coupled receptors (GPCRs) to directly inhibit airway smooth muscle (ASM) shortening, prevent (bronchoprotect) or reverse bronchoconstriction (bronchodilate); and 2) controlling airway inflammation to limit mediators that induce ASM contraction or other structural changes that impede airflow (mucus secretion, airway edema). β-2 agonists, the industry standard bronchodilator, bind to the β-2 adrenergic receptor on airway smooth muscle (ASM) and inducing $G\alpha_s$-mediated cAMP elevation and protein kinase A (PKA) activation. PKA will then limit signaling pathways

that induce ASM shortening such as intracellular calcium mobilization, rho kinase (ROCK) activation, and myosin light chain kinase (MLCK) activation. Unfortunately, about 55% of asthmatics have suboptimal control of their symptoms.

Despite β-2 agonists' ability to prevent and mitigate asthma exacerbations, they have limitations especially concerning efficacy and safety. Studies have shown that β-2 agonist use can result in adverse patient outcomes, β-2 adrenergic receptor tachyphylaxis, deterioration of asthma control, and death. New therapeutics are needed to overcome these limitations. New formulations of β-2 agonists offer improved specificity and duration of action. Unfortunately, truly novel therapeutics that overcome the limitations of existing drugs have yet to emerge.

Basic science has identified several novel targets for bronchodilation but none of these strategies have translated successfully into clinical utility. Receptor antagonists that block bronchoconstricting agonists include antagonists to the muscarinic, histamine, and leukotriene receptors to prevent shortening of ASM. Due to the multitude of bronchoconstricting mediators present in asthma pathology, however, none have been uniformly effective. Several alternative $G\alpha_s$-coupled receptors have been discovered including bitter taste receptors, the pH sensing receptor OGR1, and prostaglandin-binding EP receptors. Regrettably, these receptors lack potent and specific ligands, and have similar limitations regarding receptor tachphylaxis.

Rho Kinase and Airway Smooth Muscle

ROCK inhibitors have generated much excitement in the field. Since ROCK activation is necessary to maintain ASM tone by inhibiting MLC phosphatase, ROCK

inhibitors allow the constitutively active MLC phosphatase to de-phosphorylate MLC and allow relaxation of ASM. ROCK inhibitors blunt ASM contraction and induce bronchodilation *in vitro*, *ex vivo, and in vivo*. The limitation with targeting ROCK is that it is ubiquitously expressed, making the potential for undesired side effects high. Studies have shown that ROCK can induce hypotension as a result of vasodilation. Despite the plethora of promising findings from animal studies that demonstrate effective bronchodilation, clinical trials in humans have not been published. Inhibiting ROCK is an effective approach to induce bronchodilation, but strategies need to be developed to avoid off target effects. These strategies can include improved targeting to airway smooth muscle, or elucidating the signaling pathways that drive ROCK activation in order to identify novel mediators that are not expressed in all smooth muscle cells.

Summary

Asthma represents a syndrome that manifests as immune cell activation, inflammatory mediator release, and development of airway obstruction. PI3K, a crucial signaling molecule, plays a role in nearly all aspects of asthma pathophysiology. Inhibition of PI3K blunts mucus production, prevents mast cell degranulation, deters immune cell recruitment, and facilitates bronchodilation, all of which are therapeutically beneficial. Accordingly, PI3K represents an attractive target for the treatment of asthma.

This dissertation begins with the observation that PI3K inhibitors promote bronchodilation of human small airways (**Chapter 2**). To understand this unexpected finding, we explore the relationship of PI3K and ROCK in mediating ASM contraction, and elucidate the mechanisms by which PI3K inhibitors promote bronchodilation. We then explore the utility of PI3K inhibitors to induce bronchodilation in the contexts of

inflammation and β-2 adrenergic receptor tachyphylaxis. To further understand PI3K and ROCK signaling in ASM, we elucidate a signaling pathway leading to GPCR-mediated PI3K and ROCK activation (**Chapter 3**). Through this work, we demonstrate for the first time that $G\alpha_{12}$ mediates agonist-induced activation of PI3K and ROCK. These results reveal a novel pathway regulating PI3K and ROCK signaling that is necessary to elicit HASMC shortening. Importantly, our work has uncovered several new targets for bronchodilation and has offered new avenues for the treatment of asthma.

Figure Legends

Figure 1. Class I PI3K Signaling. Class I Phosphoinositide 3-Kinases (PI3K) are activated upon agonist binding to Receptor Tyrosine Kinases (RTK) or G-Protein Coupled Receptors (GPCR). RTKs will recruit PI3Ks to phosphorylated tyrosine residues, resulting in activation of PI3Ks. GPCRs can activate PI3Ks via G-proteins such as Gα or Gβγ, and through β-Arrestins. PI3K phosphorylates the D-3 position of the plasma membrane lipid phosphatidylinositol-4-5-bisphosphate $PI(4,5)P_2$, generating $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ will recruit proteins with pleckstrin-homology domains which include phosphoinositide-dependent kinase 1 (PDK1), AKT Kinase (AKT), Guanine Exchange Factors (GEFs), GTPase Accelerating Proteins (GAPs), Tec Family Kinases, and Non-Receptor Tyrosine Kinases. These signaling proteins will subsequently affect cellular functions including proliferation, gene expression, anti-apoptosis, cytoskeletal rearrangement, and degranulation.

Figure 2. Functions of Class I PI3K Isoforms in Cells Mediating Asthma Pathophysiology. A schematic representation of the roles of Class I Phosphoinositide 3-Kinase (PI3K) isoforms in various cell types involved in asthma pathophysiology. IgE, Immunoglobulin Type E; IL-4, Interleukin 4; IL-5, Interleukin 5; Th1, Helper T Cell Type 1; Th2, Helper T Cell Type 2.

Figures

Figure 1

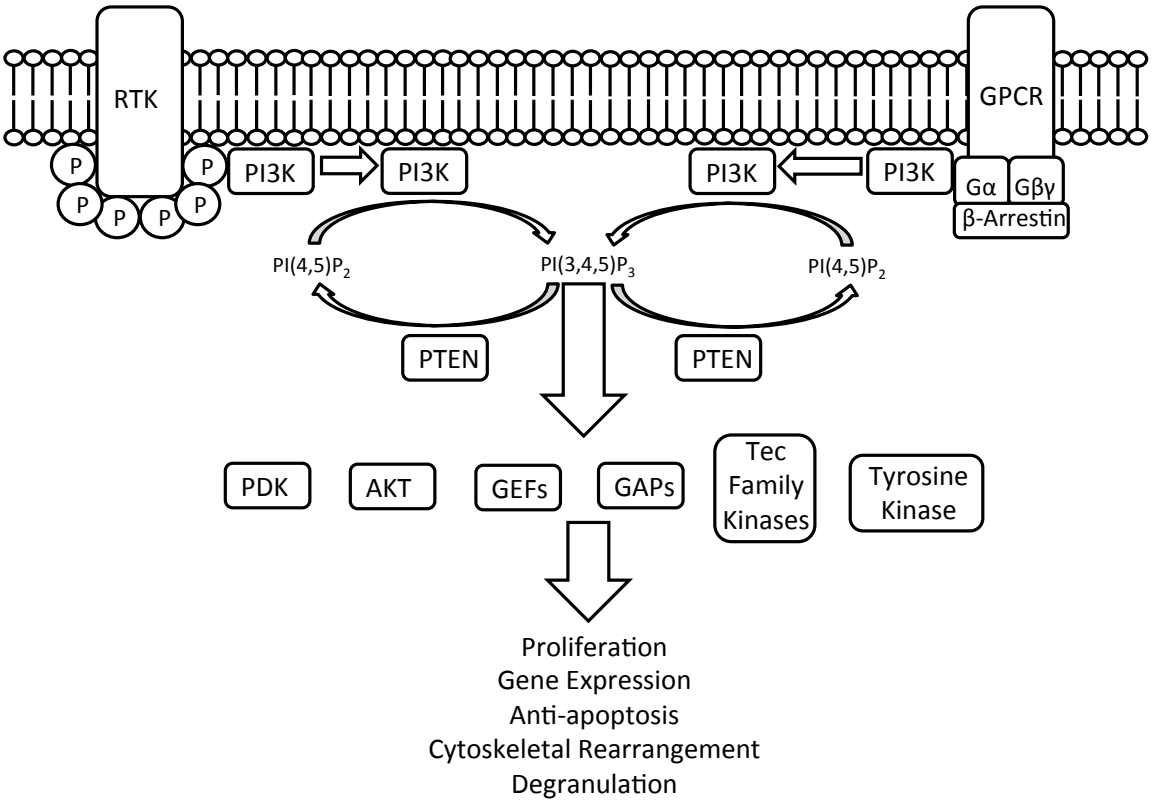

Figure 2

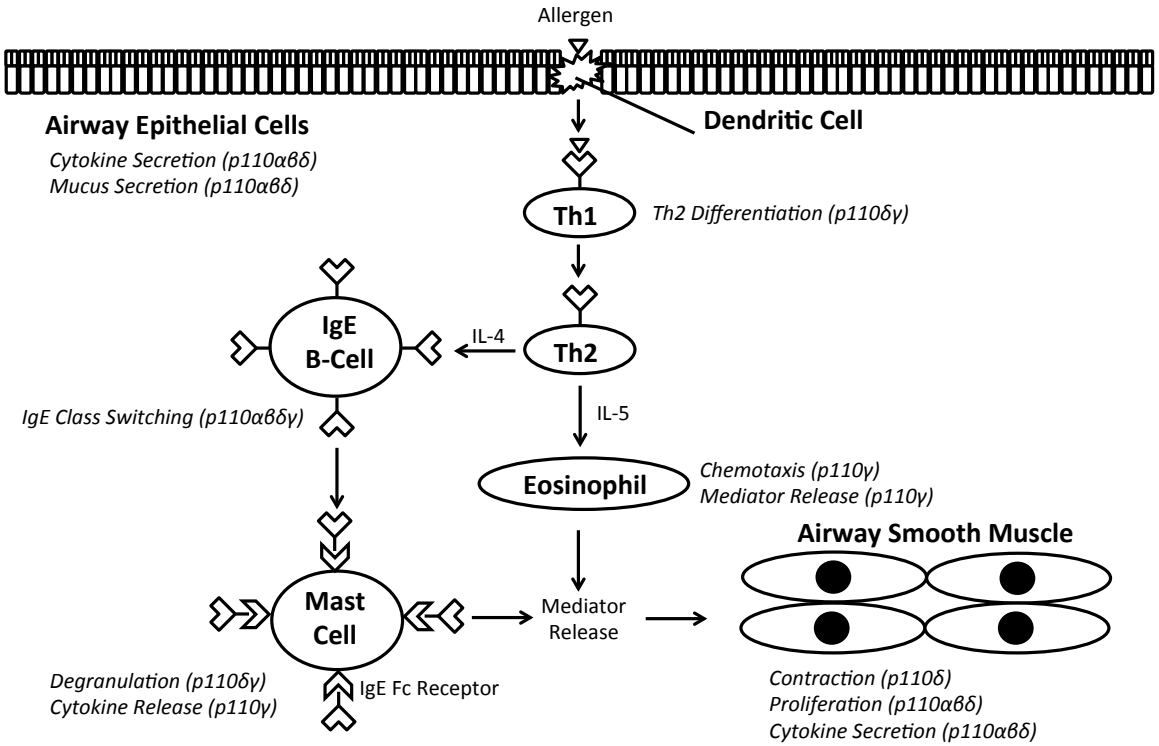
Allergen
Airway Epithelial Cells
Cytokine Secretion (p110αβδ)
Mucus Secretion (p110αβδ)
Dendritic Cell
Th1
Th2 Differentiation (p110δγ)
IgE B-Cell
IL-4
Th2
IL-5
IgE Class Switching (p110αβδγ)
Eosinophil
Chemotaxis (p110γ)
Mediator Release (p110γ)
Airway Smooth Muscle
Mast Cell
Mediator Release
Degranulation (p110δγ)
Cytokine Release (p110γ)
IgE Fc Receptor
Contraction (p110δ)
Proliferation (p110αβδ)
Cytokine Secretion (p110αβδ)

Table Legends

Table 1. Characteristics of PI3K in Asthma. ***Definition of Abbreviations:*** AHR, Airway Hyperresponsiveness; CXCL-8, C-X-C Motif Chemokine Ligand 8; IgE, Immunoglobulin Type E; IL-1, Interleukin 1; IP-10, Interferon Gamma-Induced Protein 10; MMP-1, Matrix Metallopeptidase 1; PI3K, Phosphoinositide 3-Kinase; P110α, Phosphatidylinositol 3-Kinase Catalytic Subunit Alpha Isoform; P110β, Phosphatidylinositol 3-Kinase Catalytic Subunit Beta Isoform; P110γ, Phosphatidylinositol 3-Kinase Catalytic Subunit Gamma Isoform; P110δ, Phosphatidylinositol 3-Kinase Catalytic Subunit Delta Isoform; Th2, Helper T Cell Type 2; VEGF, Vascular Endothelial Growth Factor.

Table 2. PI3K Inhibitors in Clinical Trials for Asthma and COPD. ***Definition of Abbreviations:*** PI3K, Phosphoinositide 3-Kinase; P110α, Phosphatidylinositol 3-Kinase Catalytic Subunit Alpha Isoform; P110β, Phosphatidylinositol 3-Kinase Catalytic Subunit Beta Isoform; P110γ, Phosphatidylinositol 3-Kinase Catalytic Subunit Gamma Isoform; P110δ, Phosphatidylinositol 3-Kinase Catalytic Subunit Delta Isoform;

Tables

Table 1

Table 1. Characteristics of PI3K in Asthma			
Cell/Animal	**Class I PI3K Subunits**	**Role in Asthma**	**Reference**
Mice	p110δ	AHR	33-34
	p110δ	Inflammation	36
	p110γ	Inflammation	37
	p110γ	Remodeling	37
Airway Smooth Muscle	p110δ, p110γ	Contraction	38-43
	p110α, p110β, p110δ	Proliferation	18
	p110α, p110δ	Gene Expression	43
	p110γ	β-2 Adrenergic Receptor resensitization	44
	p110δ	IL-6 secretion	45
	p110α, p110β, p110δ	VEGF expression	46
	p110α, p110β, p110δ	CXCL-8 expression	47
	p110α, p110β, p110δ	MMP-1 secretion	48
	p110α, p110β, p110δ	Proliferation	49-53
Airway Epithelial Cells	p110α, p110β, p110δ	IP-10 secretion	60
	p110α, p110β, p110δ	Nitric oxide signaling	60-61
	p110α, p110β, p110δ	Mucus secretion	63
T Cells	p110δ, p110γ	Th2 differentiation	27, 64-67
B Cells	p110α, p110β, p110δ, p110γ	IgE class-switching	68-69
Mast Cells	p110δ	Degranulation	70
	p110γ	Cytokine Release	71
Eosinophils	p110γ	Chemotaxis	72-73
	p110γ	Mediator release	74
Neutrophils	p110δ, p110γ	Migration	76
	p110α, p110β, p110δ, p110γ	Degranulation	74

Table 2

Table 2. PI3K Inhibitors in Clinical Trials for Asthma and COPD			
Compound	**Target**	**Indication**	**Clinical Trial Identifier**
Idelalisib (CAL-101)	p110δ	Chronic Lymphocytic Leukemia	FDA and EMA approved, 2014
GSK2269557	p110δ	Asthma	NCT01462617, Phase 1
IPI-145	p110δ/p110γ	Asthma	NCT01653756, Phase 2
RV-1729	p110δ/p110γ	Asthma/COPD	NCT01813084, Phase 1
AQX-1125	SHIP1 activator	COPD	NCT01954628, Phase 2

CHAPTER 2: Inhibition of PI3K promotes dilation of human small airways in a rho kinase-dependent manner

Edwin J. Yoo[1], Cynthia J Koziol-White[1], Gaoyuan Cao[1], Jie Zhang[1], Eleni Papanikolaou[1], Ivan Pushkarsky[2], Adam Andrews[2], Blanca E Himes[3], Robert D Damoiseaux[4,5], Stephen B Liggett[7], Dino Di Carlo[2,4,6], Richard C Kurten[8], and Reynold A. Panettieri Jr.[1]

[1]Rutgers Institute for Translational Medicine and Science, Child Health Institute , Rutgers University, New Brunswick, NJ, USA, [2]Department of Bioengineering, University of California, Los Angeles, CA, USA, [3]Department of Biostatistics and Epidemiology, University of Pennsylvania, Philadelphia, PA, USA, [4]California NanoSystems Institute, University of California, Los Angeles, CA, USA, [5]Department of Molecular and Medicinal Pharmacology, University of California, Los Angeles, CA, USA, [6]Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA, USA, [7]University of South Florida, Tampa, F L, USA, and [8]Arkansas Children's Hospital Research Institute and Department o f Physiology & Biophysics, University of Arkansas Medical Sciences, Little Rock, AR, USA

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Abstract

Asthma manifests as a heterogeneous syndrome characterized by airway obstruction, inflammation and hyperresponsiveness (AHR). Although the molecular mechanisms remain unclear, activation of specific PI3K isoforms mediate inflammation and AHR. We aimed to determine whether inhibition of PI3Kδ evokes dilation of airways and to elucidate potential mechanisms. Human precision cut lung slices from non-asthma donors and primary human airway smooth muscle (HASM) cells from both non-asthma and asthma donors were utilized. Phosphorylation of Akt, myosin phosphatase target subunit 1 (MYPT1) and myosin light chain (MLC) were assessed in HASM cells following either PI3K inhibitor or siRNA treatment. HASM relaxation was assessed by micro-pattern deformation. Reversal of constriction of airways was assessed following stimulation with PI3K or ROCK inhibitors. Soluble inhibitors or PI3Kδ knockdown reversed carbachol-induced constriction of human airways, relaxed agonist-contracted HASM and inhibited pAkt, pMYPT1 and pMLC in HASM. Similarly, inhibition of Rho kinase also dilated human PCLS airways and suppressed pMYPT1 and pMLC. Baseline pMYPT1 was significantly elevated in HASM cells derived from asthma donors in comparison with non-asthma donors. After desensitization of the β_2-adrenoceptors, a PI3Kδ inhibitor remained an effective dilator. In the presence of IL-13, dilation by a β agonist, but not PI3K inhibitor, was attenuated. PI3Kδ inhibitors act as dilators of human small airways. Taken together, these findings provide alternative approaches to the clinical management of airway obstruction in asthma.

Introduction

Asthma, a disorder characterized by airway inflammation and airway hyperresponsiveness (AHR), globally contributes to substantial morbidity and mortality[89]. Bronchodilators act either acutely as rescue treatment or chronically as maintenance therapy by reversing airway smooth muscle (ASM) shortening and dilating airways. While these agents are effective in preventing airflow obstruction, therapeutic limitations exist that include increasing sensitivity to bronchoconstrictive agents[90], receptor tachyphylaxis[3,4] and heterogeneity of response imparted by β_2-adrenoceptor polymorphisms[91]. Many asthma patients remain poorly controlled with bronchodilator(s), suggesting an unmet need for alternative approaches to control asthma symptoms and morbidity[91,92].

Phosphoinositide-3 kinase (PI3K), a family of kinases, phosphorylates membrane phospholipids. These kinases consist of regulatory subunits p85 and p101, and a catalytic subunit p110. Of the catalytic subunits, there are three classes that are differentially activated by receptor tyrosine kinases and GPCRs. Class I p110 catalytic subunits include α, β, γ and δ and are activated by GPCRs. Evidence suggests that PI3K activation helps drive the inflammation associated with allergic airways disease. Inhibition of PI3K attenuates allergen-induced eosinophil influx, mucus production[26], degranulation of bone marrow derived mast cells[93] and remodelling of the airways[94]. Investigators previously showed that genetic ablation or inhibition of PI3Kγ or δ isoforms substantially decreased AHR[36]. In vascular smooth muscle, inhibition of p110α attenuated membrane depolarization and contraction of aortic rings by KCl. Additionally, inhibition of PI3K class I isoforms with LY294002, wortmannin or targeted knockdown of p110α also attenuated KCl-induced phosphorylation and activation of Rho

A, myosin phosphatase target subunit 1 (MYPT1) phosphorylation and myosin light chain (MLC) phosphorylation[95]. Rho A and MYPT1 are components of the calcium sensitization pathway that amplifies contraction responses in smooth muscle. MYPT1, a phosphatase, reverses the phosphorylation of MLC required for smooth muscle shortening. Y27632, a Rho kinase inhibitor, also attenuated phosphorylation of MYPT1 while inhibition of PI3K p110γ attenuated acetylcholine-induced bronchoconstriction of murine small airways and calcium flux in murine ASM[96]. We demonstrated that inhibition of PI3Kδ also attenuated TNF-α-induced CD38 expression, an ADP-ribosyl transferase that mobilizes cellular calcium[97].

PI3K inhibition prior to stimulation with a contractile agonist attenuated smooth muscle contraction, calcium flux[44] and other contractile signalling pathways[95,98]. For the current study, we posit that PI3Kδ inhibition directly dilates human small airways. PI3K inhibition, both inhibition of class I p110 subunits [with LY294002[99]] and targeted inhibition of p110δ but not p110γ [with CAL-101[100]], attenuated carbachol-induced phosphorylation of MYPT1 and MLC in human ASM (HASM). Using human precision cut lung slices (hPCLS), we show that reversal of carbachol-induced bronchoconstriction with PI3K inhibitors or a selective rho-associated protein kinase (ROCK) inhibitor [Y27632[101]] is comparable with that induced by formoterol, a β_2-adrenoceptor agonist. Interestingly, PI3K inhibition attenuated phosphorylation of MYPT1 and MLC, but had little effect on generation of cAMP or agonist-induced calcium mobilization. We show that agonist-induced HASM contraction is reversed by both formoterol and CAL-101 to similar levels. Modulation of MYPT1 and MLC phosphorylation by PI3K inhibitors is receptor dependent, as shown by its absence following KCl stimulation. Following salmeterol -induced β_2-adrenocpetor desensitization, PI3Kδ inhibition dilates human airways more effectively than a β agonist. In a Th2 type inflammatory environment

consistent with that observed in asthma, we show that PI3K inhibitors promote airway dilation while formoterol-induced airway dilation is attenuated. Additionally, we demonstrate that MYPT1 phosphorylation is elevated in HASM derived from fatal asthma patients as compared with that derived from non-asthma patients. Taken together, these data suggest that the mechanism by which PI3K inhibition bronchodilates human airways converges at a step below an elevation in intracellular cAMP, thereby providing a complementary therapeutic strategy comparable with β agonists for reversing airflow obstruction.

Methods

Isolation and culture of HASM

HASM cells were derived from tracheas obtained from the National Disease Research Interchange (Philadelphia, PA, USA) and from the International Institute for the Advancement of Medicine (Edison, NJ, USA). HASM cell culture was performed as described previously[102]. The cells were cultured in Ham's F-12 medium supplemented with 10% FBS, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin and 2.5 mg mL^{-1}amphotericin B, and this medium was replaced every 72 h. HASM cells in subculture during passages 1–5 were used, because these cells retain the expression of native contractile protein, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin[102]. The HASM cells were derived from donors with fatal asthma or from donors who were age- and gender-matched without asthma, as shown in Supplemental Table 2.

Generation of PCLS and airway dilation assays

hPCLS were prepared as previously described[90]. Briefly, whole human lungs from non-asthma donors were dissected and inflated using 2% (w v^{-1}) low melting point agarose. Once the agarose set, the lobe was sectioned, and cores of 8 mm diameter were made. The cores that contained a small airway by visual inspection were sliced at a thickness of 350 μm (Precisionary Instruments VF300 Vibratome, Greenville, NC, USA) and collected in wells containing supplemented Ham's F-12 medium. The cores generated were randomized as to the location in the lungs they were derived from, so the slices generated came from throughout the lungs and not one specific area. This generated variation in the slices both from within a single donor, and also accounts for variation from donor to donor. Suitable airways (≤1 mm diameter) on slices were selected on the basis of the following criteria: presence of a full smooth muscle wall, presence of beating cilia and unshared muscle walls at airway branch points to eliminate possible counteracting contractile forces. Each slice contained ~98% parenchyma tissue; hence, all airways situated on a slice had sufficient parenchymal tissue to impart basal tone. Slices containing contiguous segments of the same airway served as controls and were incubated at 37°C in a humidified air-CO_2 (95–5%) incubator. Sections were rinsed with fresh media 2–3 times on days 1 and 2 to remove agarose and endogenous substances released that variably confound the production of inflammatory mediators and/or alter airway tone[90]. Airways from each core were randomized to the different treatment groups prior to the start of the experiment. Airways were constricted to a dose response of carbachol (10^{-8}–10^{-5} M), then dilated to one of the following (10^{-11}–10^{-4} M): diluent (DMSO), formoterol, isoprenaline, LY294002, CAL-101 or Y27632. DMSO alone did not induce airway dilation at the concentrations tested (data not shown).

To assess luminal area, lung slices were placed in a 12-well plate in media and held in place using a platinum weight with nylon attachments. The airway was located using a microscope (Nikon Eclipse; model no. TE2000-U; magnification, ×40) connected to a live video feed (Evolution QEi; model no. 32-0074A-130 video recorder). Airway luminal area was measured using Image-Pro Plus software (version 6.0; Media Cybernetics) and represented in mm^2 [90]. After functional studies, the area of each airway at baseline and at the end of dose the response was calculated using Image-Pro Plus software. Maximal airway dilation (E_{max}), sensitivity of the airways to contractile agonist – log of the effective concentration to induce 50% airway dilation (log EC_{50}) and the integrated response to dilatory agonist – AUC were calculated from the dose-response curves generated. The hPCLS were derived from non-asthma donors, as shown in Supplemental Table 3. Airway dilation was calculated as % reversal of maximal bronchoconstriction. Time courses of airway dilation to Y27632, CAL-101 and formoterol were performed to show the kinetics of response of the airways to reversal of carbachol-induced airway constriction by these compounds (Supplemental Figure 1).

Immunoblot analysis

HASM cells were treated with LY294002, CAL-101 or Y27632 (1 μM for time course experiments – 0–60 min, or 10^{-8}–10^{-6} M for 30 min for dose response experiments) then stimulated with carbachol (10 μM – 10 min). Cells were then treated with 500 μM perchloric acid, plates scraped and cells pelleted. Pellets were solubilized in RIPA and sonicated prior to being subjected to SDS-PAGE and transferred to nitrocellulose membranes, as previously described[103]. Phosphorylation of MYPT1, MLC and Akt were assessed, and band densities were normalized to total tubulin band density. A total of 5–7 individual donor cell lines for derived from either non-asthma or

fatal asthma donors were used in these experiments. Total protein expression of MYPT1, MLC and Akt were also assessed in the same lysates (Supplemental Figure 2).

Contractility measurements of individual HASM cells

Soft silicone elastomer films were micro-patterned with fibronectin and fluorescent fibrinogen in uniform 'X' shapes (70 μm diagonal by 10 μm thick). These substrates were prepared using a robust sacrificial approach to facilitate covalent embedding of the extracellular matrix (ECM) molecules into the film as previously described[104]. The non-patterned regions were blocked using 0.5% Pluronic F-127 preventing cell adhesion outside of the fibronectin patterns. Isolated cells adhering to these 'X'-shaped micro-patterns exerted tonic traction forces or stimulated contraction forces, resulting in deformations of the micro-patterns (Figure 3A). Dimensions of contracted micro-patterns, which correspond directly to the force applied on them by adhered cells, relative to the original unperturbed dimensions were used to assess cellular contractile responses to the tested compounds. Prior to stimulation, isolated HASM cells were seeded on the soft substrates, allowed to adhere and serum-starved for 48 h. Cells were then stimulated with bradykinin (10^{-6} M, 15 min) to induce contraction and treated with 0.5% v v^{-1} DMSO in medium (control), formoterol or CAL-101 (10^{-10}, 10^{-7} or 10^{-4} M). Each condition was performed in triplicate wells for a non-asthmatic HASM cell line. The fluorescent micro-patterns were imaged immediately before stimulation with bradykinin for a baseline reading, and approximately 15 min after administration of the treatments. Cell nuclei were stained with Hoechst 33342 prior to imaging, and only the patterns co-localized with exactly one stained nucleus were used in the analysis. Following these studies, matlab (Mathworks, Natick, MA, USA) was used to measure each individual pattern and generate distributions of the relative contractions

of the patterns for each treatment case. Using the medians of these distributions, the relative relaxation was calculated as the percent reversal from maximal contraction. Bradykinin was administered at 10^{-4} M in the movies provided in the supplemental materials, but lowered to 10^{-6} M in the experimental data shown.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology[105]. GraphPad Prism software (La Jolla, CA, USA) was used to determine statistical significance evaluated by Student's paired *t*-test for two groups or ANOVA for multiple groups. *P* values of <0.05 were considered significant. For lung slice analysis (Figures 1, 5, and 8), slices were not compared with themselves for each treatment group, so repeated measures analysis was not used. Data were found to be normally distributed, and ANOVAs were used for data analysis, with Bonferroni's post-test. Unpaired *t*-tests with Welch's correction were used after ANOVAs had established significance to compare each inhibitor with formoterol, and conditions within a given inhibitor stimulation (for example, −/+ IL-13 for LY294002-induced airway dilation). For Figure 7, results were analysed by two-way repeated measures ANOVA with desensitization pretreatment (control or salmeterol) as Factor A and bronchodilator test compound (isoprenaline or CAL-101) as Factor B. The data passed the normality, and equal variance tests and significant effects of both factors were detected. Differences were isolated using the Bonferroni *t*-test for all pairwise comparisons. Immunoblot data and single cell contractile data were analysed by Student's two-tailed *t*-tests. SigmaStat (Systat, San Jose, CA, USA) and GraphPad Prism programmes were used in statistical analyses.

Materials

Carbachol (carbamoyl choline chloride), formoterol (formoterol fumarate dihydrate), isoprenaline (ISO – isoproterenol hydrochloride), salmeterol (salmeterol xinafoate), bradykinin (bradykinin acetate salt) and perchloric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). LY294002 was purchased from Cayman Chemical Company (Ann Arbour, MI, USA), and CAL-101 was purchased from Selleck Chemicals (Houston, TX, USA). Y27632 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Rho activity assay kit was purchased from Cytoskeleton (Denver, CO, USA). Constructs (siRNA) targeting PI3K p110γ and δ were purchased from Qiagen (The Netherlands). Antibodies for detection of pMYPT1-Thr696 (5163S), pAkt (4060S), pMLC (3674S) and tubulin (3873S) were purchased from Cell Signalling Technologies (Danvers, MA, USA). Pharmacological properties of the inhibitors utilized in these studies are found in Supplemental Table 1.

Results

PI3K inhibition reverses carbachol-induced constriction of human small airways in a dose-dependent manner

To determine if inhibition of PI3K dilates precontracted small human airways, PCLS were prepared from donors with no history of lung disease. PCLS airways were treated with carbachol to induce bronchoconstriction and then treated with increasing doses of LY294002, CAL-101 or formoterol to evaluate airway dilation (Figure 1). Both LY294002 and CAL-101 markedly reversed carbachol-induced bronchoconstriction, but

it was not to the same level as to the β agonist formoterol. Further, we evaluated ROCK and PI3K inhibitor-induced airway dilation and detected responses as early as 5–10 min, which were sustained through 30 min following a single dose of inhibitor (Supplemental Figure 1). LY294002 and CAL-101 also reversed bronchoconstriction induced by histamine (data not shown). Pretreatment with these inhibitors had little effect on carbachol-induced calcium transients in HASM cells (Supplemental Figure 3). These data indicate that inhibition of the p110 subunit of PI3K and specifically of p110δ is bronchodilatory and that the mechanism likely involves events distal to the activation of GPCRs mediating bronchoconstriction in human airways.

PI3K inhibition attenuates carbachol-induced phosphorylation of Akt, MYPT1 and MLC

Phosphorylation of key components of excitation-contraction coupling pathways, including Akt (a surrogate to measure PI3K activation), MYPT1 and MLC mediate agonist-induced HASM shortening. Conversely, dephosphorylation of these targets promotes lengthening of HASM. Accordingly, carbachol-induced activation (phosphorylation) of Akt, MYPT1 and MLC were examined in HASM in the presence and absence of PI3K inhibition. As shown in Figure 3, selective PI3Kδ inhibition with CAL-101 (Figure 2A) or a pan-PI3K inhibitor LY294002 in HASM cells (Figure 2B) inhibited carbachol-induced phosphorylation of Akt, MYPT1 and MLC in a time- (Figure 2A and B) and dose-dependent manner (Figure 2C). To assess whether membrane depolarization-induced phosphorylation of Akt, MYPT1 and MLC required PI3K activation, HASM cells were treated with KCl in presence or absence of CAL-101 (Figure 2D). Unlike carbachol-induced phosphorylation of MYPT1 and MLC, KCl-dependent phosphorylation of MYPT1 and MLC was unaffected by CAL-101 while phosphorylation

of Akt was PI3K-dependent. These data suggest that the mechanism for smooth muscle contractile responses to membrane depolarization by KCl is either parallel or it converges below PI3K and MYPT1 phosphorylation on phosphorylation of MLC.

PI3K inhibition reverses bradykinin-induced shortening of single HASM cells

To determine if inhibition of PI3K reverses contractile agonist-induced shortening of single HASM cells, cells were seeded on fluorescently micro-patterned soft silicone elastomer substrates, stimulated with bradykinin to induce shortening and then treated with increasing doses of CAL-101 or formoterol. Relaxation of the cells was evaluated, and we show that CAL-101 reversed bradykinin-induced shortening comparable with maximal levels induced by formoterol Figure 3.

Suppression of PI3K p110δ protein expression inhibits –carbachol-induced phosphorylation of MYPT1 and MLC

Murine models showed that genetic ablation of PI3K p110γ or δ attenuated airway hyperreactivity after allergen challenge[36,44]. We demonstrated that carbachol-induced phosphorylation of contractile proteins is attenuated by pharmacological inhibition of PI3K p110, specifically p110δ. We next sought to define the requirement for p110δ in modulating phosphorylation events mediating HASM shortening by using siRNA targeting PI3K p110δ to suppress protein expression in HASM cells (Figure 4A). As a consequence, carbachol-induced phosphorylation of both MYPT1 and MLC were significantly reduced (Figure 4B). However, knockdown of PI3K p110γ had little effect on phosphorylation of MYPT1 and MLC (Supplemental Figure 4). Knockdown of either PI3K p110δ or p110γ reduced carbachol-induced Akt phosphorylation, but this was not

statistically significant. The limitation to these observations is that although we detected expression of PI3Kδ, we could not detect PI3Kγ in HASM. Taken together, pharmacological inhibition and siRNA-mediated suppression indicate that p110δ likely mediates distal events regulating ASM shortening in response to carbachol.

Inhibition of rho kinase with Y27632 induces airway dilation of human small airways and attenuates activation of rho kinase and phosphorylation of MLC

In vascular smooth muscle, PI3K p110α regulates Rho activation and MYPT1 phosphorylation[95]. Rho kinase (ROCK) activation plays a role in the maintenance of airway muscle tone in guinea pig ileum[106] and pig trachea[107] strips. To determine if ROCK activation similarly mediates maintenance of airway tone in carbachol bronchoconstricted PCLS airways, we treated hPCLS with increasing doses of the ROCK inhibitor Y27632 and observed that Rho kinase inhibition evoked airway dilation similar to that observed with the β agonist formoterol, but not quite as robust. Like LY294002 and CAL-101, Y27632 induced dose-dependent airway dilation (Figure 5A). ROCK inhibition also attenuated carbachol-induced phosphorylation of MYPT1 and MLC, but not Akt in HASM cells (Figure 5B and C). To define the role for ROCK in receptor-independent contraction, HASM cells were also treated with KCl in the presence or absence of Y27632. As shown in Figure 5D, inhibition of ROCK activity attenuated receptor-independent phosphorylation of MYPT1 and MLC. These data indicate that while Rho kinase activation by carbachol occurs either downstream or parallel to PI3K activation, both converge on MYPT1 and MLC phosphorylation.

Baseline rho kinase activity is increased in HASM derived from asthma donors

The present studies point to MYPT1 phosphorylation as an important mechanism mediating a procontractile phenotype. As compared with normal donors, HASM cells derived from asthma donors retain a phenotype consistent with asthma manifested by hypersecretion of inflammatory mediators, growth and proliferation[85,108–112]. Therefore, we evaluated MYPT1 activation in HASM cells from non- and fatal asthmatic donors. As shown in Figure 6A, HASM cells derived from asthma donors exhibit greater baseline phosphorylation of MYPT1 in comparison with HASM cells derived from non-asthma donors. In parallel, we found that ROCK activity levels were also elevated in HASM cells derived from asthma donors (Figure 6B). These data suggest that there is a greater activation of Rho kinase-dependent pathways evoking MYPT1 phosphorylation in HASM cells from donors with asthma.

PI3K and rho kinase inhibitors induce airway dilation after β_2AR desensitization or IL-13 stimulation

To determine the potential benefit of pharmacological inhibition of PI3K and ROCK as 'rescue' bronchodilators, we evaluated responses of hPCLS desensitized to a β agonist or maintained in a Th2-like inflammatory environment, elevated levels of IL-13. Tachyphylaxis of the β_2-adrenoceptor limits the therapeutic efficacy of β agonists in airways disease[113,114]. Because this involves a proximal event (failure to activate of Gsα), we hypothesized that PI3K inhibition would induce airway dilation despite β_2-adrenoceptor tachyphylaxis. In hPCLS treated for 24 h with salmeterol, isoprenaline failed to reverse carbachol-induced bronchoconstriction (Figure 7), implying tachyphylaxis. By contrast, CAL-101 effectively dilated airways despite impaired β_2-

adrenoceptor activation. Inhibition of PI3K alone or in combination with isoprenaline had no direct effect on generation of cAMP in HASM (Supplemental Figure 5).

We previously demonstrated that IL-13, an important Th2 inflammatory mediator in asthma, attenuates β_2-adrenoceptor agonist-induced airway dilation[90], but has little effect on airway dilation to the TAS2 receptor agonist chloroquine[115]. Accordingly, we posited that IL-13 may limit PI3K inhibitor-mediated airway dilation of carbachol-constricted hPCLS. While formoterol-induced airway dilation was attenuated by IL-13 treatment, PI3K inhibitor-induced airway dilation was not affected significantly by IL-13 (Figure 8). Taken together, these experiments using hPCLS define new therapeutic approaches to bronchodilation.

Discussion

Asthma, a chronic respiratory disorder marked by inflammation, recurrent reversible airflow obstruction and AHR, remains a significant cause of morbidity and mortality[89]. Despite efficacy of both long- and short-acting bronchodilators, a significant number of asthma patients remain uncontrolled due to a number of factors including tachyphylaxis of the receptor[3,4], genetic polymorphisms altering receptor function or heterogeneity of patient responses[91,92]. There exists an unmet need for alternative methods to induce bronchodilation that are independent of the β_2-adrenoceptor.

Studies in murine models of allergen challenge showed a role for class I PI3K p110γ and δ isoforms in trafficking/activating inflammatory cells, decreasing mucus production and attenuating AHR following allergen challenge[26,93,94]. Interestingly, inhibition of PI3K p110δ, either by genetic ablation[26] or by pretreatment with a δ selective pharmacological inhibitor[44], had little effect on baseline methacholine-induced

bronchoconstriction *in vivo* or in *ex vivo* murine lung slices. A PI3Kγ selective inhibitor attenuated acetylcholine-induced bronchoconstriction of murine precision cut lung slices and calcium flux in murine ASM cells[44]. We now show that pretreatment of HASM cells with either LY294002 or CAL-101 had little effect on calcium flux in HASM cells (Supplemental Figure 3). We provide evidence of PI3K p110δ, but not γ, isoform expression in ASM[97] [Supplemental Figure 2], validating our approach to selectively targeting PI3K p110δ in modulation of contractile pathways in HASM and airway dilation in PCLS. In addition to using human cells and PCLS, our study also focused on using PI3K inhibitors to induce airway dilation rather than on using PI3K inhibition as a bronchoprotective agent. We show that in human airways, inhibition of PI3K p110 isoforms with LY294002, as well as selective inhibition of p110δ with CAL-101, promotes airway dilation comparable with the β agonists isoprenaline or formoterol. Clinically, the onset of action for formoterol is within 1–4 min following inhalation[116]. In contrast, airway dilation in response to a PI3K or ROCK inhibitor is slower given these compounds act on intracellular targets rather than a cell surface receptor. We demonstrate that the time scale of inhibitor-induced dilation of carbachol-constricted airways is in agreement with the time course of PI3K or ROCK inhibition of carbachol-induced phosphorylation of Akt, MYPT1 and MLC (Figures 1, 2, and 5) and Supplemental Figure 1. As a surrogate for airway constriction and relaxation, we utilized a novel *in vitro* approach measuring contractility of single HASM cells to assess the effect of PI3K inhibition on cellular dynamics of contraction and relaxation (Figure 3A). Using this system, we show that both formoterol and CAL-101 induce relaxation of HASM cells that have been precontracted by bradykinin in a dose-dependent manner (Figure 3B). In this system, we show that there is no significant difference in the maximal relaxation elicited by formoterol or CAL-101. These data suggest that CAL-101 is as effective of a HASM

relaxant as formoterol at a maximal concentration. These data provide alternative mechanisms/targets for inducing airway dilation in humans that are independent of β_2-adrenoceptor activation.

In vascular smooth muscle, the PI3K p110α isoform regulates Rho activation and phosphorylation of MYPT1 and MLC in response to KCl[95]. Others demonstrate that in response to histamine, phosphorylation of MLC in vascular smooth muscle is PI3K-dependent[98]. To determine potential mechanisms by which inhibition of PI3K induces airway dilation, we examined carbachol-induced phosphorylation of Akt (as a marker of PI3K activation), MYPT1 and MLC. Carbachol-induced Akt, MYPT1 and MLC phosphorylation was inhibited by CAL-101 and LY294002 in a time- and dose-dependent manner. To assess the contribution of PI3K p110 isoforms to non-receptor-mediated activation of contractile pathways, we also stimulated HASM cells with KCl in the presence and absence of pharmacological inhibition of PI3K. We demonstrated that phosphorylation of MLC and MYPT1 are independent of PI3K activation in response to KCl. These data provide a mechanism by which carbachol-induced bronchoconstriction is reversed by PI3K inhibition, but that receptor-independent stimulation of contractile pathways with KCl is PI3K-independent unlike in vascular smooth muscle[98]. The physiological relevance of KCl-induced phosphorylation of Rho kinase and MLC remains unclear[117]. Additionally, we demonstrated that knockdown of PI3K p110δ attenuates carbachol-induced phosphorylation of MLC and MYPT1. We showed that PI3K p110δ, but not γ, is expressed in HASM cells and that inhibition of PI3K isoforms has no effect on expression of levels of total Akt, MYPT1 or MLC proteins. Additionally, we demonstrated that knockdown of PI3Kγ in HASM cells has little effect on carbachol-induced phosphorylation of Akt, MYPT1, and MLC. Given the lack of specific antibodies, we cannot completely rule out the possibility that the PI3K p110γ isoform may also play

role in relaxation of ASM, but given the data we have concerning lack of its expression in isolated smooth muscle, we feel that this is unlikely.

β-adrenoceptor agonists induce bronchodilation by generating cAMP and activating protein kinase A that inhibit, in part, MLC kinase and phosphorylation of MLC. To determine whether PI3K inhibition affects the phosphorylation status of MLC through generation of cAMP, we treated HASM cells with LY294002 and CAL-101 in the presence or absence of isoprenaline and assessed cAMP generation (Supplemental Figure 5). We showed that treatment with the PI3K inhibitors has little effect on cAMP generation and that pretreatment with the inhibitors prior to isoprenaline stimulation also had little effect. These data suggest that PI3K inhibitor-induced airway dilation is independent of inducing or potentiating β_2 agonist-induced cAMP production. Additionally, we showed that PI3K inhibition does not appear to directly affect carbachol-induced calcium transients in HASM (Supplemental Figure 3).

Given evidence in vascular smooth muscle linking PI3K and Rho pathways[118], we sought to determine whether Rho kinase inhibition attenuated constriction of human small airways and excitation-contraction pathways activated in HASM cells. We found that Y27632, a Rho kinase inhibitor, reversed carbachol-induced bronchoconstriction to levels comparable with that observed using PI3K inhibitors (Figure 5A). We also showed that phosphorylation of MYPT1 and MLC, but not Akt, are inhibited by the ROCK inhibitor Y27632 in a time- and dose-dependent manner (Figure 5B and C). In assessing the effect of Rho kinase inhibition on receptor-independent activation of contractile pathways, however, we found that Y27632 attenuated KCl-dependent phosphorylation of MYPT1 and MLC (Figure 5D). These data suggest that receptor-dependent and receptor-independent contractile pathways activate ROCK and MLC but that receptor-dependent

pathways also activate PI3K that requires Rho kinase to mediate contractile responses, as illustrated in Figure 9.

Given both clinical[92] and experimental evidence[113] demonstrating agonist-induced desensitization of the β_2-adrenocpetor and tachyphylaxis, we posited that inhibition of contractile pathways downstream from the receptor and independently of the β_2-adrenoceptor-mediated pathways would induce airway dilation. While pretreatment of PCLS with salmeterol diminished airway dilation to isoprenaline, CAL-101 retained the ability to dilate human small airways despite impaired β_2-adrenoceptor activation (Figure 7). While we presume that this is due to receptor desensitization, it is could also be a manifestation of the antagonistic properties of residual lipophilic salmeterol remaining in the tissue despite washout[119,120]. Carbachol-induced airway constriction in these slices were comparable between the salmeterol-treated and non-treated airways, indicating that the bronchoprotective properties of salmeterol treatment were not retained after washing of the tissue.

The inflammatory milieu in asthma engenders AHR to contractile agonists and attenuates β_2 agonist-induced bronchodilation[47,90,121]. Previously, we showed that exposure to IL-13 renders airways hyposensitive to β_2-adrenoceptor agonist-mediated airway dilation but not TAS2 receptor-mediated airway dilation[115]. Our data show that in the presence of IL-13, PI3K inhibitor-dependent airway dilation retained efficacy while β_2 agonist-mediated airway dilation was partially inhibited. Farghaly *et al.* demonstrated that IL-13 augmented carbachol-induced contractility of murine tracheal rings and that this hyperresponsiveness was mediated by PI3K p110 isoforms, specifically PI3K p110δ[45]. We previously reported that IL-13 augments carbachol-induced airway narrowing and attenuates β_2-adrenoceptor agonist-induced airway dilation in human PCLS[90,115]. Although IL-13 attenuates β_2-adrenoceptor agonist-induced airway[90,115,121–123], IL-13 has

little effect on PI3K p110 inhibitor-induced airway dilation as shown in Figure 8. We and others have shown that HASM cells derived from fatal asthma donors manifest distinct phenotypes compared to cells derived from non-asthmatic patients, including cell proliferation[110], enzymatic function[124,125], receptor signalling and sustained calcium transients consistent with contractility of the muscle[108,111,126]. We now show that phosphorylation of MYPT1, a phosphatase that regulates MLC phosphorylation and HASM cell shortening, is elevated at baseline in asthma-derived HASM cells (Figure 6). Phosphorylation of MYPT1 inactivates this key signalling event of calcium sensitization. We also demonstrated that endosomes from asthma-derived HASM cells, as compared with non-asthma HASM cells, manifest increased PI3Kγ activation and a reduction in protein phosphatase 2A activity that inhibits β_2-adrenoceptor resensitization[47]. Collectively, these findings have implications providing a plausible hypothesis as to why β_2 agonists are less effective in Th2 airway inflammation[127]. Moreover, our data suggest that in a Th2 inflammatory milieu promoting AHR, PI3K inhibitors retain efficacy as bronchodilators while β agonist responses are attenuated.

Our data suggest that both PI3K and ROCK inhibitors induce airway dilation. Because others have suggested that PI3K inhibitors may also act as anti-inflammatory agents in asthma, PI3K activation may serve as a unique therapeutic target in asthma and chronic obstructive pulmonary disease to attenuate airway inflammation and promote airway dilation.

Figure Legends

Figure 1. PI3K inhibition reverses carbachol-induced bronchoconstriction in a dose-dependent manner in hPCLS. Airways were preconstricted to carbachol (10^{-8}–10^{-4} M) prior to dilation to LY294002, CAL-101 or formoterol (10^{-11}–10^{-5} M). Maximal airway dilation (Emax, ANOVA, $P = 0.03$) and AUC ($P = 0.004$) for each inhibitor was significantly different than formoterol-induced dilation. Data are representative of $n \geq 5$ donors, 26–33 slices per condition, with bars representing mean + SEM.

Figure 2. PI3K inhibition blocks carbachol-induced MYPT1 and MLC phosphorylation in HASM cells. Time-dependent inhibition of carbachol-induced (10 min, 10 μM) MYPT1 and MLC phosphorylation in HASM cells by CAL-101 (A) and LY294002 (B) (1 μM). Data are representative of five different HASM donors ($n = 5$ donors, mean ± SEM). *$P < 0.05$. P values are time points compared with basal. (C) Concentration-dependent inhibition of carbachol-induced MYPT1, Akt and MLC phosphorylation in HASM cells by LY294002 and CAL-101. Data are representative of five different HASM donors. *$P < 0.05$. P values compared with basal ($n = 5$, mean ± SEM). (D) CAL-101 (1 μM) prevents carbachol- (10 min, 10 μM).

Figure 3. PI3K inhibition reverses bradykinin-induced shortening in isolated HASM cells. Cells were stimulated to contract with bradykinin (10^{-6} M) prior to treatment with 0.5% v v^{-1} DMSO control, or formoterol or CAL-101(10^{-10}, 10^{-7} and 10^{-4} M). (A) Representative images of fibronectin micro-patterns on ultra-soft silicone elastomer films. Top: typical uncontracted and contracted micro-patterns. HASM F-actin is shown in green. Bottom: averaged patterns for each treatment case generated from one pattern taken from each of the nine imaging sites used per sample. (B)

Quantification of cell relaxation to formoterol or CAL-101 (10^{-10}, 10^{-7} and 10^{-4} M) following bradykinin contraction. Bars represent mean of the triplicates + SEM, with each column representing ≥720 cells analysed per triplicate well measurement from a single donor. $P < 0.05$ was considered significant.

Figure 4. siRNA knockdown of PI3K p110δ prevents carbachol-induced (10 min, 10 μM) MLC phosphorylation (pMLC) by suppression of ROCK in HASM cells. (A) Immunoblot analyses of HASM cells transfected with PI3K p110δ siRNA or scrambled siRNA. (B) Inhibition of carbachol-induced phosphorylation of MYPT1 (pMYPT1) and MLC (pMLC) in HASM cells by PI3K p110δ siRNA. Data are representative of five independent experiments ($n = 5$, mean + SEM); $^*P < 0.05$.

Figure 5. Rho Kinase inhibition induces airway dilation in hPCLS and inhibits carbachol-induced MYPT1 and MLC phosphorylation in HASM cells. (A) hPCLS were bronchoconstricted with carbachol ($n = 12$ donors) and sequentially treated with 0.02% DMSO (Veh) or increasing concentrations of Y27632 (10^{-11} – 10^{-5} M). Data are representative of $n \geq 5$ separate donors (two-tailed t-test $P = 0.02$ for Emax, $P = 0.001$ for AUC). (B) Time-dependent inhibition of carbachol-induced (10 min, 10 μM) phosphorylation of MYPT1 (pMYPT1) and MLC (pMLC) in HASM cells by Y27632 (1 μM). Data are representative of five different HASM donors ($n = 5$, mean + SEM). $^*P < 0.05$. P values are time points compared with vehicle control. (C) Concentration-dependent inhibition of carbachol-induced phosphorylation of MYPT1 and MLC in HASM cells by Y27632 (30 min pretreatment, 10^{-8}–10^{-6} M). Data are representative of five independent experiments. $^*P < 0.05$. P values compared with vehicle control. (D) Y27632 (1 μM) prevents carbachol- (10 min, 10 μM) and KCl- (10 min, 50 mM) mediated pMYPT1 and pMLC, but has little effect on Akt

phosphorylation in primary HASM cells. Data are representative of five different HASM donors (n = 5, mean + SEM).

Figure 6. Rho kinase activation is greater in HASM cells obtained from subjects with asthma as compared to those of non-asthmatic subjects. HASM cells obtained from fatal asthmatic and non-asthmatic subjects were lysed and subjected to immunoblot for phosphorylated MYPT1 (A), or elisa to measure ROCK activation (B). Data represent mean + SEM fold change of MYPT1 phosphorylation as compared with tubulin control (immunoblotting) and fold compared with basal (for elisa).
*P < 0.05. n = 7 asthma and seven non-asthma for (A). n = 5 asthma and seven non-asthma for (B).

Figure 7. PI3K inhibition induces airway dilation of small airways despite desensitization of the β_2-adrenoceptors. hPCLS from four separate donors 79–81 slices/condition, total of 160 slices, were incubated in the presence or absence of 50 nM salmeterol (SALM; 24 h) to induce desensitization of the β_2-adrenoceptor. After washout and treatment with carbachol, isoprenaline (ISO)-induced airway dilation was significantly attenuated by salmeterol pretreatment. CAL-101-induced airway dilation, however, was indistinguishable from control pretreatment and was enhanced compared with the previous treatment of β agonist-desensitized airways. Data are presented as mean + SEM *P < 0.05.

Figure 8. PI3K inhibition induces airway dilation of small airways despite inflammation. PI3K and ROCK inhibitor-induced airway dilation in the presence and absence of IL-13. hPCLS were incubated in the presence or absence of IL-13

(100 ng·mL^{-1}, 18 h), precontracted to ~60–70% of baseline luminal area and relaxed to a dose response of LY294002, CAL-101 or the β_2-adrenoceptor agonist formoterol (10^{-11}–10^{-5} M). AUC was compared between control and IL-13 treatment for each compound used to dilate the airways. Data are representative of $n \geq 4$ donors, 5–44 slices per condition, with bars representing mean + SEM, with $P \leq 0.05$ considered significant (ANOVA $P = 0.01$; two-tailed *t*-tests: control versus IL-13 for formoterol $P = 0.009$, control versus IL-13 for CAL-101 P = n.s., control versus IL-13 for LY294002 P = n.s.).

Figure 9. An overview of the signalling mechanisms underlying PI3K and ROCK inhibitor-mediated airway dilation of HASM cells. IP_3, inositol triphosphate; RyR, ryanodine receptor; MLCK, MLC kinase.

Figures

Figure 1

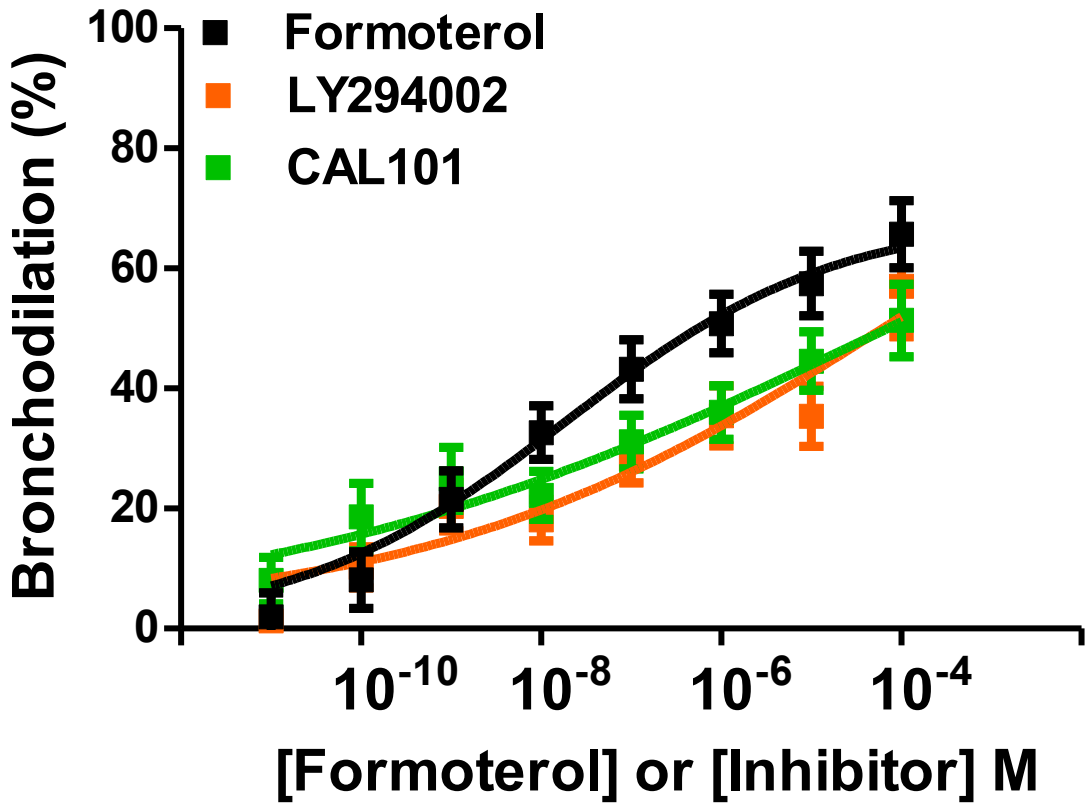


Figure 2

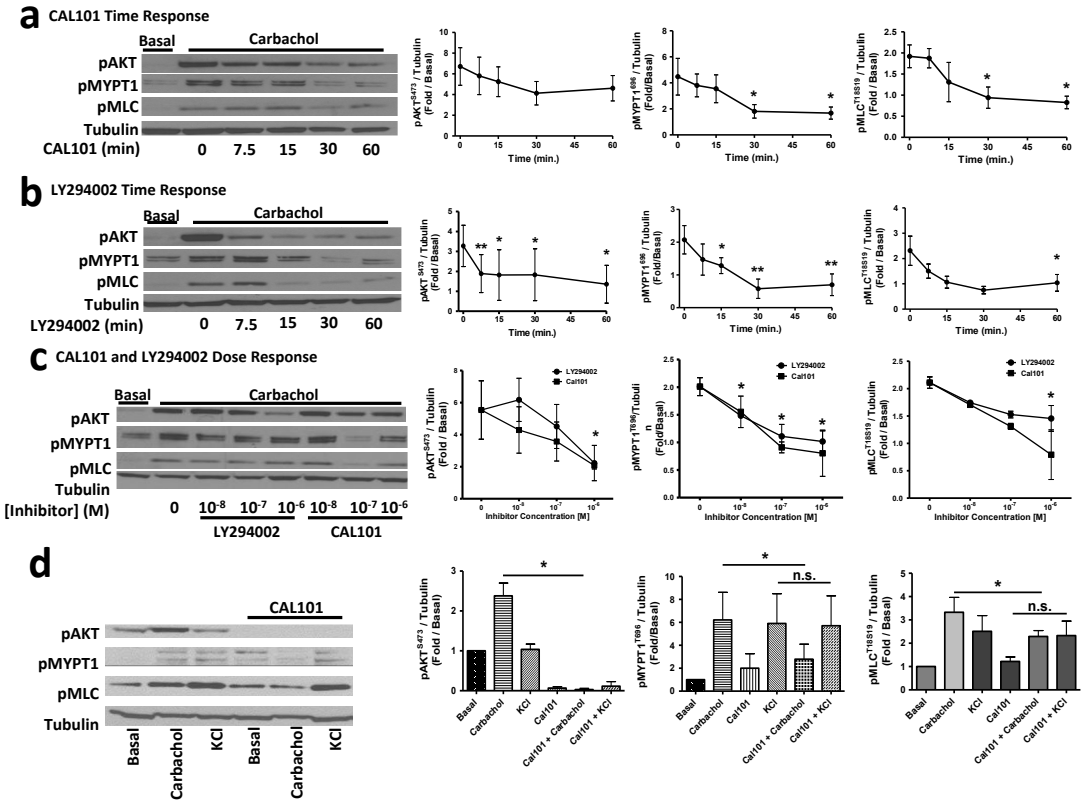

a
CAL101 Time Response
Basal
Carbachol
pAKT
pMYPT1
pMLC
Tubulin
CAL101 (min)
0 7.5 15 30 60
b
LY294002 Time Response
LY294002 (min)
c
CAL101 and LY294002 Dose Response
[Inhibitor] (M)
LY294002
CAL101
d
Basal
Carbachol
KCl
Time (min.)
Inhibitor Concentration [M]
n.s.

Figure 3

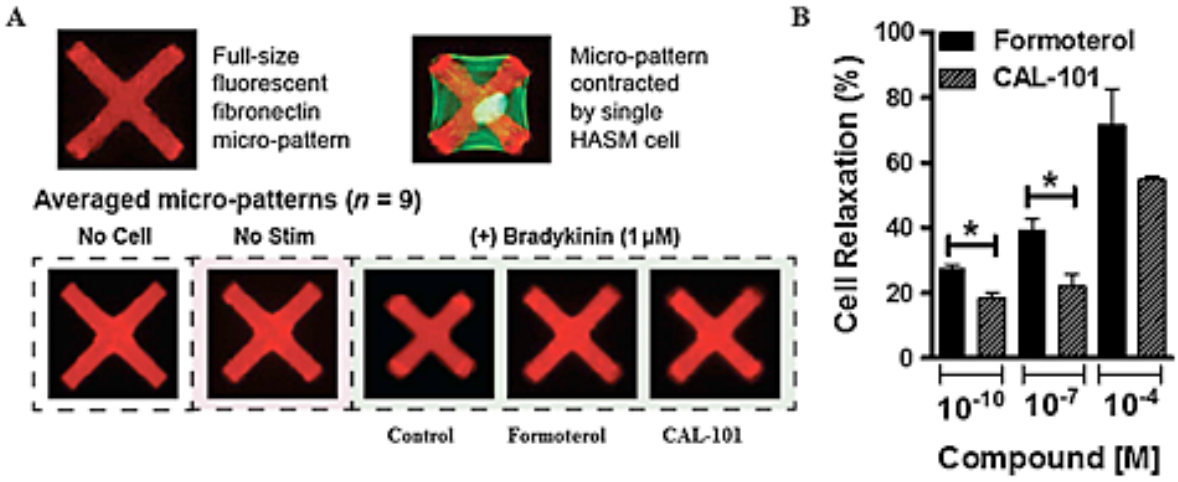

A
Full-size fluorescent fibronectin micro-pattern
Micro-pattern contracted by single HASM cell
Averaged micro-patterns (n = 9)
No Cell
No Stim
(+) Bradykinin (1 μM)
Control
Formoterol
CAL-101
B
Formoterol
CAL-101
Cell Relaxation (%)
100
80
60
40
20
0
*
*
10-10
10-7
10-4
Compound [M]

Figure 4

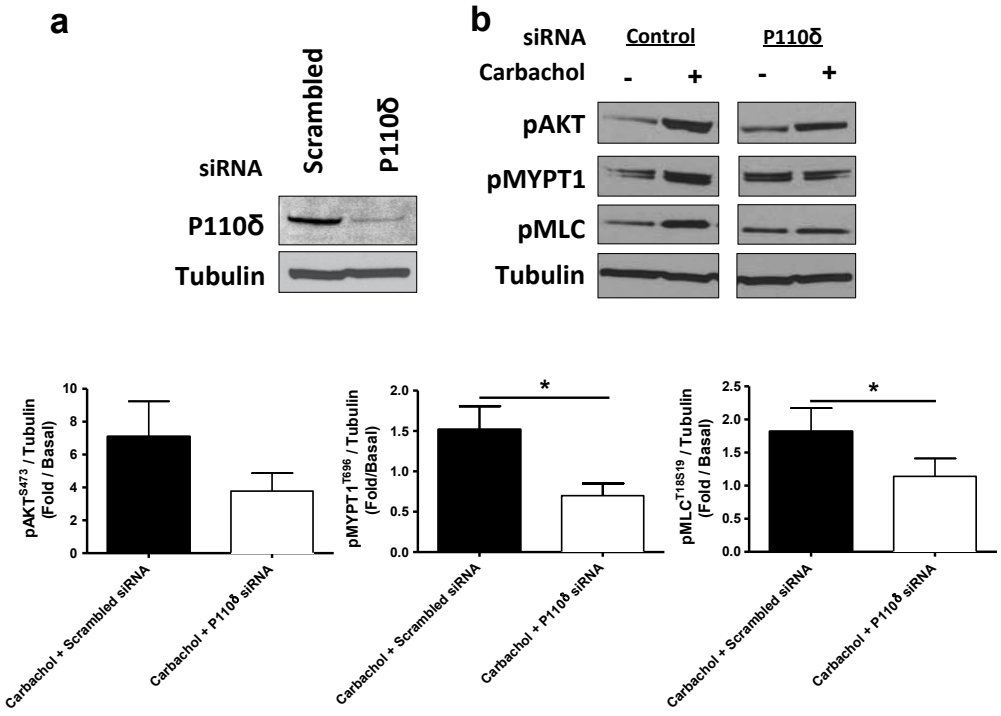

a
siRNA
Scrambled
P110δ
P110δ
Tubulin
b
siRNA
Control
P110δ
Carbachol
- + - +
pAKT
pMYPT1
pMLC
Tubulin
pAKT^S473 / Tubulin (Fold / Basal)
pMYPT1^T696 / Tubulin (Fold/Basal)
pMLC^T18S19 / Tubulin (Fold / Basal)
*
*
Carbachol + Scrambled siRNA
Carbachol + P110δ siRNA

Figure 5

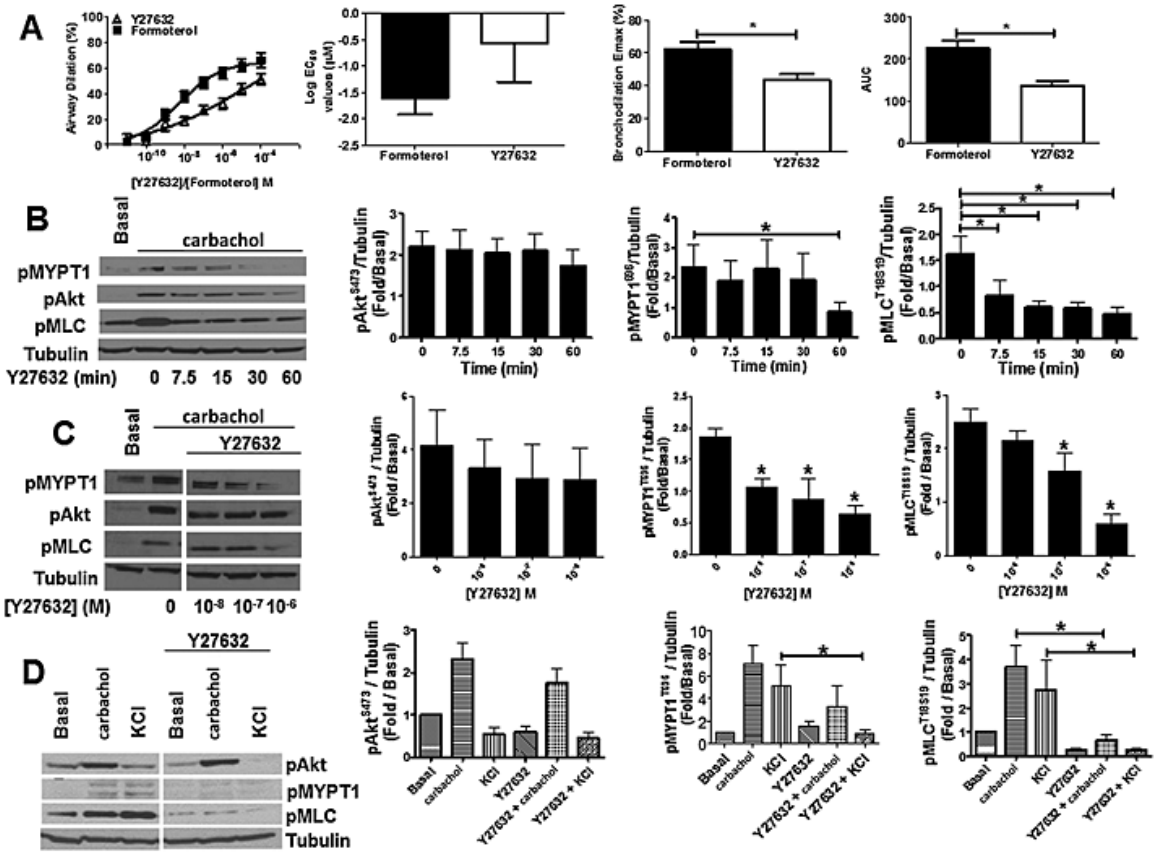

A
Y27632
Formoterol
Airway Dilation (%)
[Y27632]/[Formoterol] M
Log EC50 values (uM)
Formoterol
Y27632
Bronchodilation Emax (%)
AUC
B
Basal
carbachol
pMYPT1
pAkt
pMLC
Tubulin
Y27632 (min) 0 7.5 15 30 60
Time (min)
C
Basal
carbachol
Y27632
[Y27632] (M) 0 10-8 10-7 10-6
[Y27632] M
D
Basal
carbachol
KCl
Y27632
Y27632 + carbachol
Y27632 + KCl

Figure 6

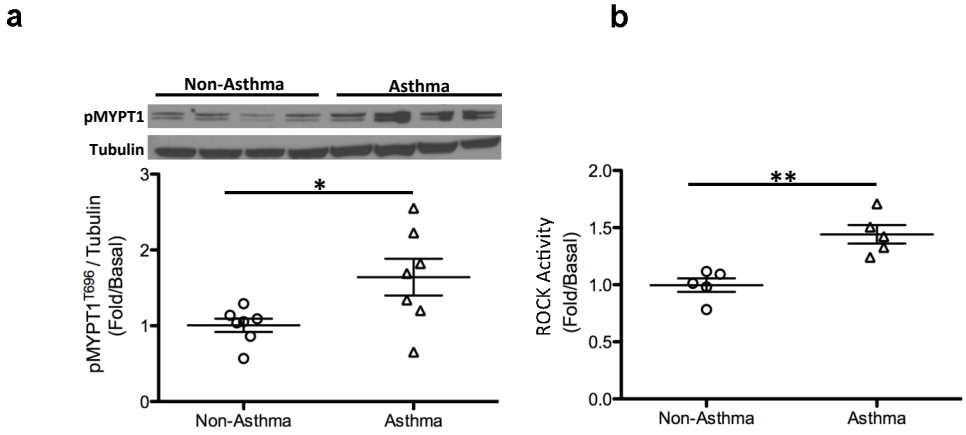

a
b
Non-Asthma
Asthma
pMYPT1
Tubulin
pMYPT1T696 / Tubulin (Fold/Basal)
*
Non-Asthma
Asthma
ROCK Activity (Fold/Basal)
**
Non-Asthma
Asthma

Figure 7

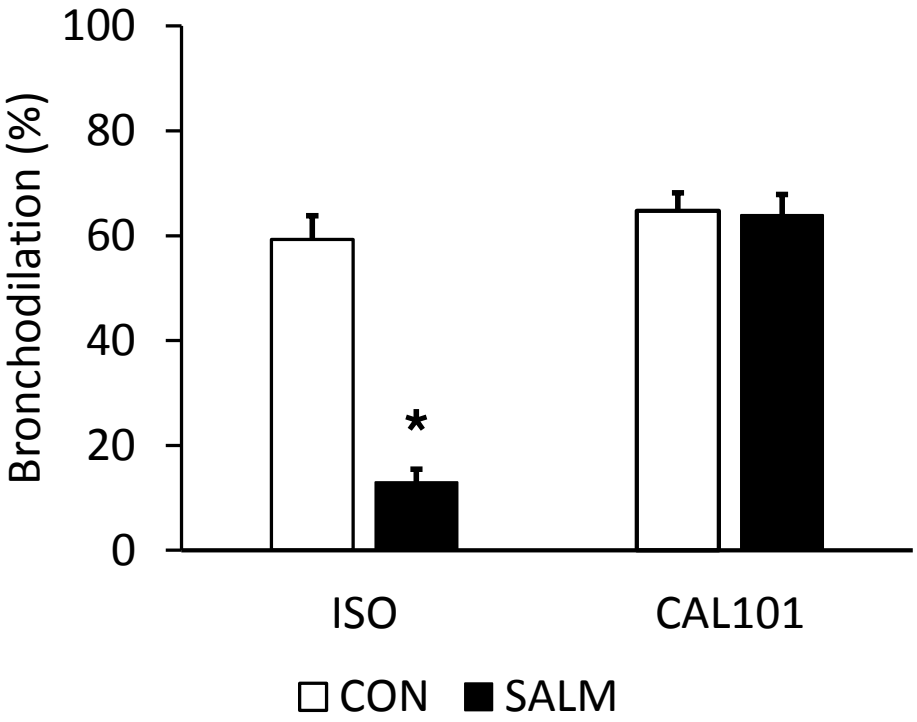


Figure 8

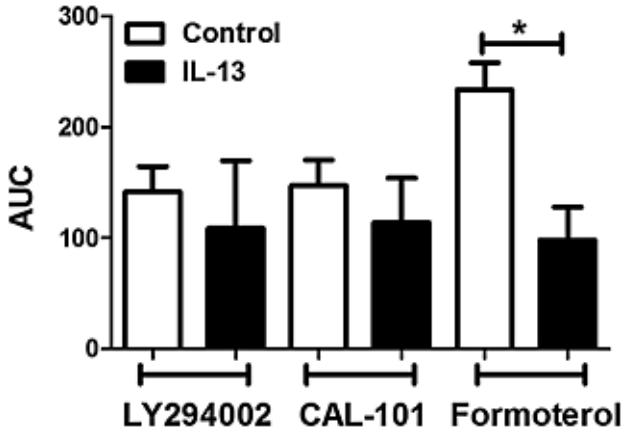
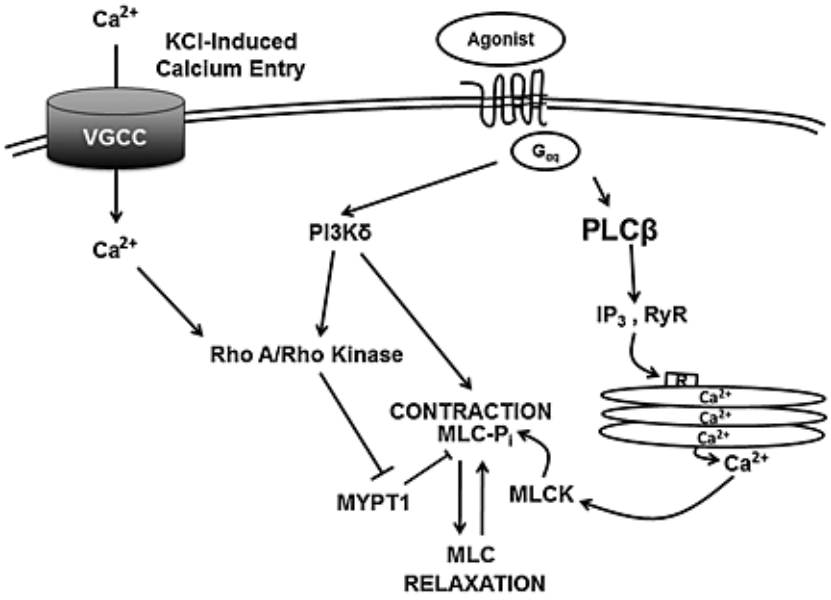


Figure 9


Ca2+
KCl-Induced
Calcium Entry
Agonist
VGCC
Gαq
Ca2+
PI3Kδ
PLCβ
IP3 , RyR
Rho A/Rho Kinase
R
Ca2+
Ca2+
Ca2+
CONTRACTION
MLC-Pi
Ca2+
MYPT1
MLCK
MLC
RELAXATION

Supplemental Figure Legends

Supplemental Figure 1. Time course of airway dilation to CAL-101 and Y27632. Airways in lung slices were preconstricted to carbachol, then dilated to CAL-101 or Y27632 (60 μM), 0–30 min. Data is representative of mean ± SEM for 4–15 slices/condition.

Supplemental Figure 2. PI3K p110δ (A) and γ (B) mRNA expression in airway smooth muscle assessed by RNAseq. PI3K inhibition has little effect on expression of total protein levels (C) of MYPT1 (D), Akt (E), and MLC (F). Data is representative of n = 5–8 (A and B) or n = 2 (C-F) HASM cell lines.

Supplemental Figure 3. PI3K inhibition has little effect on agonist-induced calcium mobilization. HASM were stimulated with 10 μM carbachol following a 30 min pretreatment with vehicle (DMSO), CAL-101 or LY294002 (1 μM) and single cell calcium transients measured using Fluo-8 calcium sensing dye. (A) Time course tracings of calcium flux. Both peak calcium (B) and AUC (C) for each treatment were plotted as mean ± SD.

Supplemental Figure 4. siRNA knockdown of PI3K p110γ had little effect on carbachol-induced (10 min, 10 μM) phosphorylation of MLC (pMLC), MYPT1, or Akt in HASM cells. (A) Immunoblot analyses of HASM cells transfected with PI3K p110γ siRNA or scrambled siRNA. 3 (B) Slight inhibition of carbachol-induced phosphorylation of Akt (pAkt) and MYPT1 (pMYPT1) in HASM cells by PI3K p110δ

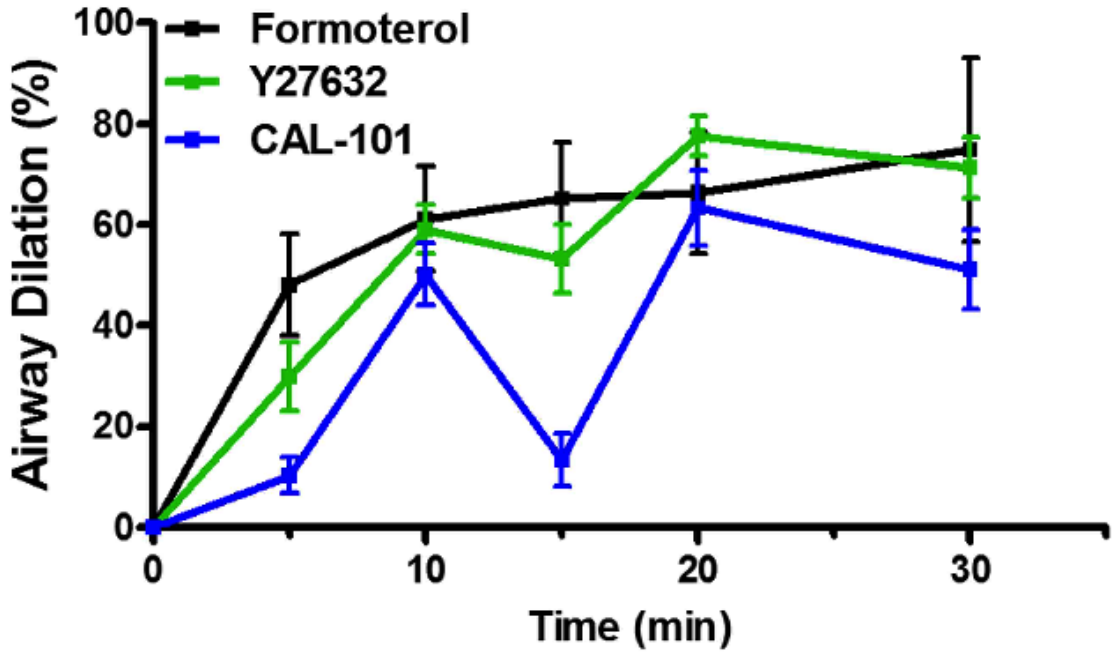
siRNA. Data are representative of five independent experiments (n = 5, mean + SEM); *P < 0.05.

Supplemental Figure 5. PI3K inhibition has little effect alone to induce or augment cAMP levels. Data are representative of 3 donors and each condition in triplicate, with bars representing mean ± SEM with a P value of <0.05 considered significant.

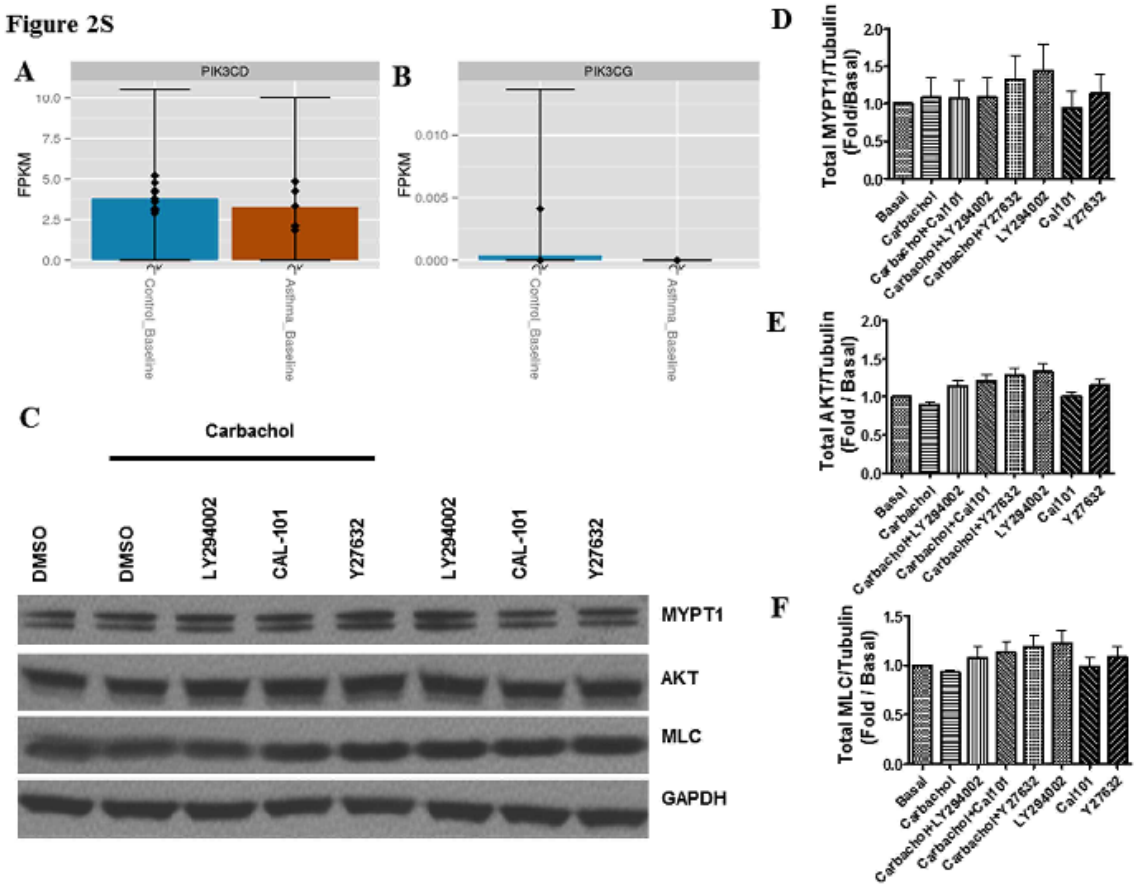
Supplemental Figures

Supplemental Figure 1

Figure 1S

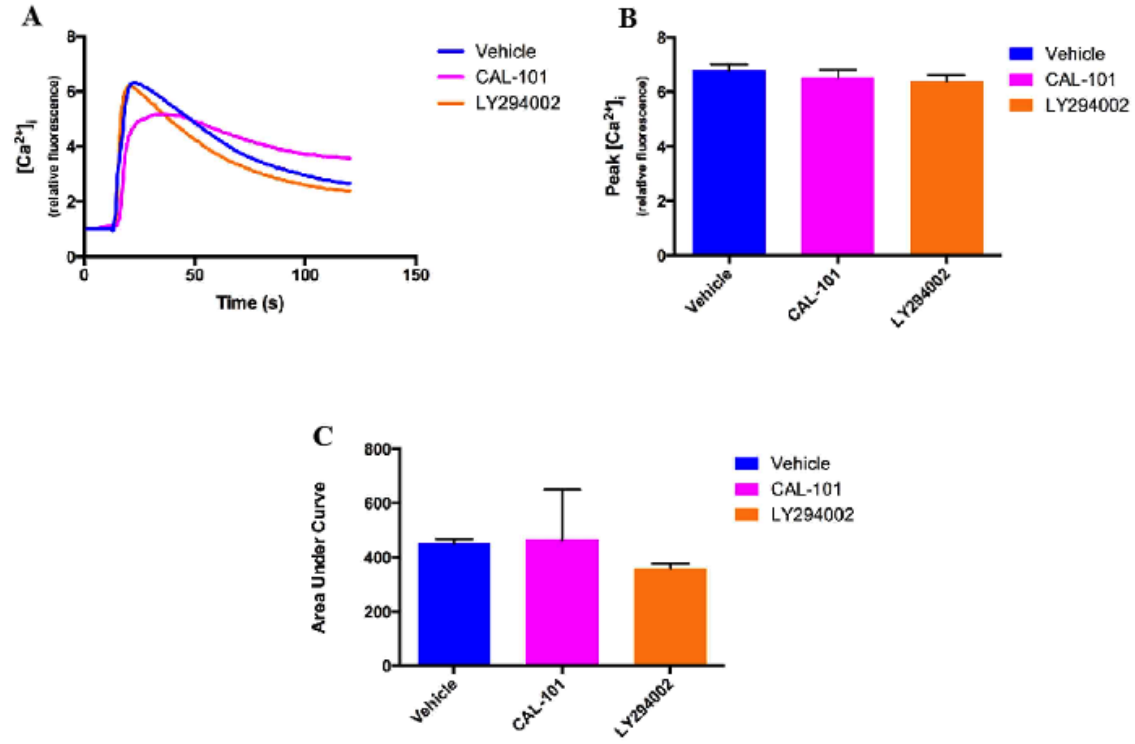


Supplemental Figure 2

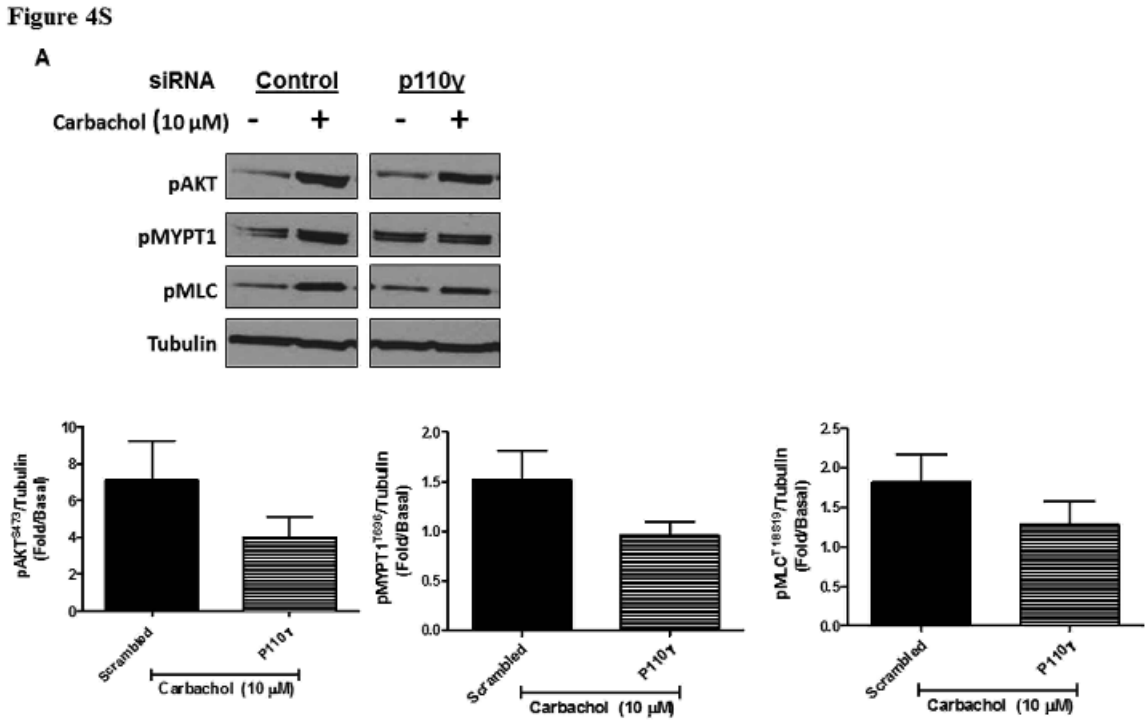


Supplemental Figure 3

Figure 3S

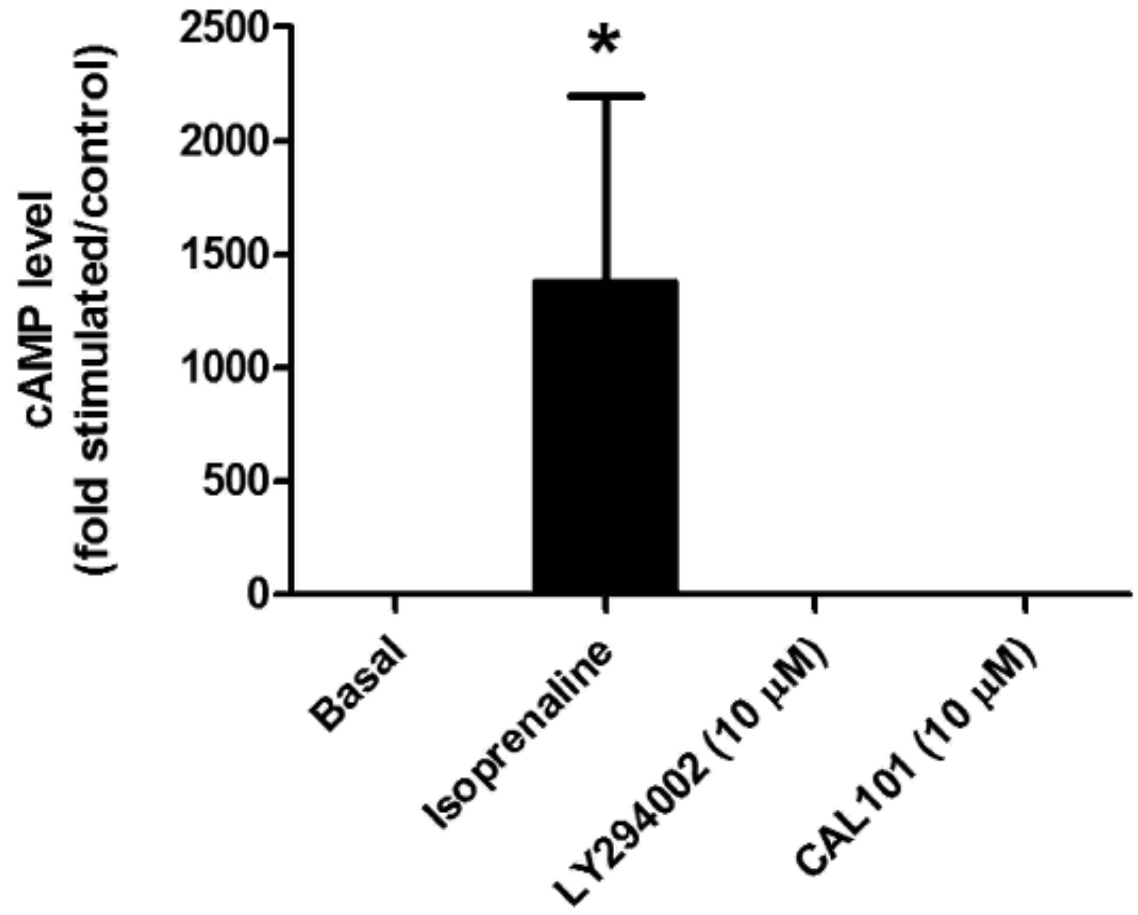


Supplemental Figure 4



Supplemental Figure 5

Figure 5S



Supplemental Table Legends

Supplemental Table 1. Pharmacologic properties of the inhibitors utilized in this study. *In vitro* kinase assay results, IC_{50}values in cell based assays, and the corresponding literature citations are shown for CAL-101, LY294002, and Y27632.

Supplemental Table 2. Donor demographics of non-asthma hPCLS donors.

Supplemental Table 3. Donor demographics of asthma and non-asthma HASM cell donors.

Supplemental Tables

Supplemental Table 1

Table 1S

Inhibitor	IC_{50} (kinase assay)	IC_{50} in cells/tissue	Citation
CAL-101	p110α = 820 nM	pAKT following PDGF stimulation EC_{25} = 10 μM; pAKT following LPA stimulation EC_{50} = 1.9 μM in fibroblasts	Lannutti BJ *et al.* (2011) Blood, 117:591-594.
	p110β = 565 nM		
	p110γ = 2.5 nM		
	p110δ = 89 nM		
LY294002	PI3K = 1.4 μM	BrdU uptake in aortic smooth muscle = 32 μM	Vlahos CJ *et al.* (1994) JBC, 269:5241-5248.
Y27632	ROCK1 Ki = 0.22 μM	G1-S phase cell cycle progression in 3T3 cells = between 10 and 100 μM	Ishizaki *et al.* (2000) Mol Pharmacol, 57:976-983.
	ROCK2 Ki = 0.30 μM		

Supplemental Table 2

Table 2S

Age	Race	Gender	Cause of Death	Asthma
48	Caucasian	Female	Asthma	Yes
59	Caucasian	Female	Asthma	Yes
15	Hispanic	Female	Asthma	Yes
31	African American	Male	Asthma	Yes
44	Hispanic	Male	Asthma	Yes
44	Caucasian	Female	Asthma	Yes
9	Caucasian	Male	Asthma	Yes
50	Caucasian	Male	Stroke	No
28	Asian	Male	Head Trauma	No
22	Caucasian	Female	Head Trauma	No
28	African American	Female	Stroke	No
45	Caucasian	Male	Overdose	No
28	African American	Male	Gun shot wound	No
24	Caucasian	Female	Stroke	No
52	Caucasian	Female	Stroke	No
21	Caucasian	Male	Gun shot wound	No

Supplemental Figure 3

Table 3S

Age	Race	Gender	Cause of Death
54	Caucasian	Male	Stroke
20	African American	Female	Stroke
54	Hispanic	Male	Stroke
22	Caucasian	Male	Head Trauma
52	Caucasian	Male	Head Trauma
41	Hispanic	Male	Anoxia
53	Caucasian	Male	Anoxia
50	African American	Male	Anoxia
28	Caucasian	Male	Anoxia
17	Hispanic	Male	Head Trauma
55	Caucasian	Female	Head Trauma
22	Caucasian	Male	Head Trauma
40	African American	Male	Stroke
49	African American	Male	Stroke
49	Caucasian	Male	Stroke
34	African American	Female	Stroke
38	Caucasian	Male	Head Trauma
14	Hispanic	Female	Head Trauma

CHAPTER 3: $G\alpha_{12}$ Facilitates Methacholine-Induced Shortening in Human Airway Smooth Muscle By Modulating Phosphoinositide 3-Kinase-Mediated Activation In A RhoA-Dependent Manner

Edwin J. Yoo[1], Gaoyuan Cao[1], and Reynold A. Panettieri Jr.[1]

[1]Rutgers Institute for Translational Medicine and Science, Child Health Institute , Rutgers University, New Brunswick, NJ, USA,

Abstract

Asthma manifests as airway hyperresponsiveness and inflammation. Importantly, G-protein-coupled receptors (GPCRs) regulate contraction of the human airway smooth muscle cell (HASMC), the pivotal cell regulating bronchomotor tone. Although PI3K-dependent activation of Rho Kinase is necessary for HASMC contraction and PI3K inhibition promotes bronchodilation of human small airways, the upstream mechanisms driving GPCR-mediated PI3K/ROCK axis activation remain unclear. Since G12 family proteins regulate smooth muscle tone by activating Rho Kinase, we hypothesize that $G\alpha_{12}$ may regulate HASMC contraction by activation of the PI3K/ROCK axis. We used siRNA and pharmacological tools, as well as HASMCs overexpressing p115RhoGEF-RGS proteins that limit M3R-mediated activation of $G\alpha_{12}$ in order to determine the role in modulating PI3K/ROCK axis activation and HASMC contraction. Our data showed that knockdown of the M3R attenuated carbachol-induced activation of AKT, MYPT1, and MLC phosphorylation. We also showed that $G\alpha_{12}$ coimmunoprecipitated with the M3R, and that p115RhoGEF-RGS expression limited carbachol-mediated induction of SRE-luciferase reporter. $G\alpha_{12}$ siRNA attenuated carbachol-induced activation of AKT, MYPT1, and MLC phosphorylation, and that p115RhoGEF-RGS overexpression limited carbachol-induced activation of AKT. Furthermore, we demonstrated that siRNA and pharmacological inhibition of RhoA blunted carbachol-mediated activation of PI3K, and that RhoA inhibitors induced dilation of hPCLS, implicating RhoA as a pivotal mediator of airway tone. Our data demonstrate a novel coupling of the M3R to $G\alpha_{12}$ HASMCs and that $G\alpha_{12}$ plays a crucial role in contraction through RhoA-dependent activation of the PI3K/ROCK axis. We

show that inhibition of RhoA induces bronchodilation in hPCLS, and that targeting $G\alpha_{12}$ signaling may elucidate novel therapeutic targets in asthma.

Introduction

Airway Hyperresponsiveness (AHR), a hallmark of asthma, represents exaggerated airway narrowing in response to contractile agonists such as acetylcholine[128,129]. Human airway smooth muscle cells (HASMCs) mediate AHR by shortening in response to contractile agonists[130]. Additionally, HASMCs is the primary target of bronchodilators, a cornerstone in the management of asthma[39].

Acetylcholine release from postganglionic parasympathetic nerves innervating the airway activates the M3-muscarinic acetylcholine receptor (M3R), a G protein-coupled receptor expressed by HASMCs [131]. Stimulation of the M3R evokes $G\alpha_{q/11}$-mediated calcium release from the sarcoplasmic reticulum, inducing in MLC Kinase (MLCK) activation and myosin light chain (MLC) phosphorylation. MLC phosphorylation induces actomyosin cross-bridge cycling and HASMC shortening[132]. In parallel, activation of Rho Kinase (ROCK) by the small GTPase RhoA, phosphorylates and inactivates MLC Phosphatase (MLCP). Inhibition of the constitutively active MLCP de-phosphorylates MLC, that sustains MLC activation and modulates HASMC tone[133,134].

We recently demonstrated that phosphoinositide 3-kinase (PI3K), a lipid kinase, is a necessary mediator of muscarinic receptor-induced ROCK activation and human airway bronchoconstriction, and that PI3K inhibitors can reverse carbachol-induced bronchoconstriction by attenuating PI3K/ROCK-axis activation[43]. The therapeutic importance of ROCK signaling is emphasized by the ability of ROCK and PI3K inhibitors promote bronchodilation. The upstream mechanisms regulating muscarinic receptor-induced ROCK and PI3K activation in HASMC remain unclear and may provide insight into novel targets for asthma therapy[135].

$G\alpha_{12/13}$ family members, including $G\alpha_{12}$ and $G\alpha_{13}$, promote ROCK signaling by activating Rho Guanine Nucleotide Exchange Factors (RhoGEFs) including p115RhoGEF, that exchange GDP for GTP and activate RhoA[136]. p115RhoGEF contains a Regulator of G Protein Signaling (RGS) domain, that specifically limits $G\alpha_{12/13}$ signaling after activation[137]. $G\alpha_{12/13}$ proteins mediate various cell functions including stress fiber formation, cytoskeletal rearrangement, and proliferation[138,139]. In the context of HASMC function, however, $G\alpha_{12/13}$ signaling remains poorly understood. In rodents, $G\alpha_{12/13}$ proteins contribute to AHR and have elevated expression upon allergen challenge[140,141]. HASMCs express multiple GPCRs that are known to couple to $G\alpha_{12/13}$ proteins, including PAR, thromboxane, and EDG receptors[139]. The M3R has also been shown to couple to $G\alpha_{12/13}$ proteins in HEK293 cells[142] but no studies in HASMCs have confirmed $G\alpha_{12/13}$ coupling to the M3R. Challenges in employing molecular approaches in primary cells, as well as the lack of pharmacological inhibitors against $G\alpha_{12/13}$ proteins, provide obstacles in characterizing the role of $G\alpha_{12/13}$ proteins in HASMC[143].

In this study, we sought to determine the contribution of $G\alpha_{12/13}$ proteins in muscarinic receptor-induced PI3K/ROCK axis activation in HASMCs. To study the contribution of $G\alpha_{12/13}$ proteins in muscarinic receptor signaling, we used siRNA transfection in primary HASMCs and newly developed inhibitors that are highly specific to RhoA[144]. Additionally, we generated HASMC cell lines that overexpress the RGS domain of p115RhoGEF, effectively limiting $G\alpha_{12/13}$ signaling. Our data show that $G\alpha_{12}$ couples to the M3R in HASMCs, and that $G\alpha_{12}$ plays an important role in facilitating HASMC shortening by promoting PI3K-mediated activation of ROCK in a RhoA-dependent manner.

Methods

Materials

CHRM2 (L-005463-01-0005), CHRM3 (L-005464-00-0005), NT siRNA (D-001810-10-05), GNA12 (L-008435-00-0005), GNA13 (L-009948-00-0005), RhoA (L-003860-00-0005), and Rac1 (L-003560-00-0005) siRNA were obtained from Dharmacon (Lafayette, CO, USA). Carbachol (carbamoyl choline chloride), formoterol (formoterol fumarate dihydrate), isoprenaline (ISO – isoproterenol hydrochloride), bradykinin (bradykinin acetate salt), pertussis toxin and perchloric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Rhosin (555460) was purchased from EMD Millipore (Darmstadt, Germany). Antibodies for detection of pMYPT1-Thr696 (5163S), pAkt (4060S), pMLC (3674S) and GAPDH (2118S), total AKT (4691S) were purchased from Cell Signalling Technologies (Danvers, MA, USA). Antibodies for immunoprecipitation and detection of the M3 receptor (SC-9108) and $G\alpha_{12}$ (SC-409) were obtained from Santa Cruz Biotechnology. Total MLC antibody (MABT180) was obtained from EMD Millipore (Darmstadt, Germany). Total MYPT1 antibody (612165) was obtained from BD Biosciences.

Isolation and Culture of HASMC

Human lungs were received from the National Disease Research Interchange (Philadelphia, PA, USA) and from the International Institute for the Advancement of Medicine (Edison, NJ, USA) and HASM cells were derived from the tracheas. All cell lines and tissue are obtained from de-identified donors and does not constitute human subject research as described by the Rutgers Institutional Review Board. Culture of HASM cells was conducted as described previously[145]. Briefly, cells were cultured in

Ham's F-12 medium supplemented with 100 U mL^{-1} penicillin, 0.1 mg mL^{-1}, streptomycin, 2.5 mg mL^{-1} amphotericin B and 10% FBS. Medium was replaced every 72h. HASM cells were only used during subculture passages 1-4 due to the strong expression of native contractile proteins[102]. In pertussis toxin studies, cells were treated with 1 μg/ml of pertussis toxin for 18h. All pharmacologic inhibitors were used with DMSO as the vehicle at a final concentration of 0.1% and were used to treat HASMC 30 min prior to agonist stimulation.

Retroviral Infection

Stable expression of GFP and p115rhogefRGS-GFP was achieved by retroviral infection as described previously [146,147]Briefly, retrovirus for the expression of each construct was produced by cotransfecting GP2–293 cells with pVSV-G vector (encoding the pantropic (VSV-G) envelope protein) and pLPCX-GFP or pLPCX-p115rhogefRGS-GFP. Forty-eight hours after transfection, supernatants were harvested and used to infect human telomerase reverse transcriptase (hTERT) immortalized airway smooth muscle cultures, with effective virus concentrations established by immunoblot analysis. Cultures were selected to homogeneity with 1 μg mL^{-1} puromycin as described previously[146,147].

Generation of hPCLS and Airway Dilation Assays

hPCLS were prepared as previously described[90]. Briefly, human lungs were dissected and filled with 2%(w v^{-1}) low melting point agarose. After the agarose solidified, the lobe was sectioned and 8 mm diameter cores were generated. Cores containing small airways were sliced at a thickness of 350 μM using Precisionary Instruments VF300 Vibratome. They were then collected in supplemented Ham's F-12

medium. Generated slices came from all areas of the lung and not just one specific area. Airways from each core were randomized to the different treatment groups prior to the start of the experiment. Airways were constricted to a dose response of carbachol (10^{-8}-10^{-5} M), then dilated to one of the following (10^{-11}-10^{-4} M): diluent (DMSO), formoterol, or rhosin. DMSO alone did not induce airway dilation at the concentrations tested (data not shown).

To assess luminal area, lung slices were placed in a 12-well plate in media and held in place using a platinum weight with nylon attachments. The airway was located using a microscope (Nikon Eclipse; model no. TE2000-U; magnification, ×40) connected to a live video feed (Evolution QEi; model no. 32-0074A-130 video recorder). Airway luminal area was measured using Image-Pro Plus software (version 6.0; Media Cybernetics) and represented in mm^2[90]. After functional studies, the area of each airway at baseline and at the end of dose the response was calculated using Image-Pro Plus software. Maximal airway dilation (E_{max}), sensitivity of the airways to contractile agonist – log of the effective concentration to induce 50% airway dilation (log EC_{50}) and the integrated response to dilatory agonist – AUC were calculated from the dose-response curves generated. Airway dilation was calculated as % reversal of maximal bronchoconstriction.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. GraphPad Prism software (La Jolla, CA, USA) was used to determine statistical significance evaluated by Student's paired *t*-test for two groups or ANOVA for multiple groups. *P* values of <0.05 were considered significant. For lung slice analysis, slices were not compared with themselves for each

treatment group, so repeated measures analysis was not used. Data were found to be normally distributed, and ANOVAs were used for data analysis, with Bonferroni's post test. Unpaired *t*-tests with Welch's correction were used after ANOVAs had established significance to compare each inhibitor with formoterol, and conditions within a given inhibitor stimulation (for example, –/+ IL-13 for LY294002-induced airway dilation). Results were analysed by two-way repeated measures ANOVA with desensitization pretreatment (control or salmeterol) as Factor A and bronchodilator test compound (isoprenaline or CAL-101) as Factor B. The data passed the normality, and equal variance tests and significant effects of both factors were detected. Differences were isolated using the Bonferroni *t*-test for all pairwise comparisons. Immunoblot data and single cell contractile data were analysed by Student's two-tailed *t*-tests. SigmaStat (Systat, San Jose, CA, USA) and GraphPad Prism programmes were used in statistical analyses.

siRNA transfection

Ham's F-12 media, DharmaFECT 1 reagent, and siRNA were combined in a microcentrifuge tube according to manufacturer's protocol and incubated for 20 min. HASMCs were trypsinized and trypsin was inactivated with 5% FBS. Cells were centrifuged and resuspended in Ham's F-12 media. Cell suspension was added to siRNA mixture and incubated for 15 min. Cell suspension and siRNA mixture was then seeded into cell culture plates according to experimental design and incubated for 6h. After 6h, complete cell culture media (described above) was added to the cell culture plate wells in a 1:1 ratio and was incubated for 18h. After 18h, media was changed to complete media. Cells were serum-deprived for 24h before collection. Cells were collected 72h post-transfection.

cAMP Assay

HASMCs were seeded in a 24 well plate until about 80% confluent and serum-deprived overnight. Cells were stimulated and lysed using cAMP-Screen System ELISA from Applied Biosystems (Bedford, MA, USA). Experiment was conducted according to manufacturer's protocol.

SRE-Luciferase Assay

HASMC were seeded and grown to 75% confluence. Complete medium was removed and Cignal Lenti SRE Reporter (CLS-010L-1) was added to cells with SureENTRY Transduction reagent (336921) according to manufacturer's protocol. After 24 h, media was changed to complete medium. After 24 h, media was changed to serum-free media. Following 48h incubation in serum-free media, cells were stimulated with carbachol for 6h and collected with luciferase lysis buffer (E1483) from Promega (Madison, WI, USA).

Immunoblot Analysis

After transfection with siRNA or incubation with pharmacologic inhibitors, cells were stimulated with carbachol (10 μM – 10 min). Perchloric acid was added to cell media to attain a final concentration of 0.1%. Cells were scraped, collected, and pelleted. Pellets were washed once with ice cold PBS. PBS was aspirated and pellets were solubilized in RIPA. Sample buffer was added and samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes, as previously described[43,103]. Phosphorylation of MYPT1, MLC and AKT were assessed, and band densities were normalized to GAPDH, total MYPT1, total MLC, or total AKT band density.

Co-immunoprecipitation

After stimulation, HASMCs grown on 10 cm plates were lysed using ice-cold cell lysis buffer from Cell Signaling Technology (Danvers, MA, USA) containing 1% Triton X-100 with Protease and Phosphatase Inhibitors from Thermo Fisher Scientific (Waltham, MA, USA). Lysate was incubated with primary antibody and incubated overnight with gentle rocking at 4°C. Protein A was incubated with lysates with gentle rocking for 3 hours at 4°C. Samples were microcentrifuged for 30 seconds at 4°C. Pellet was washed five times with cell lysis buffer. Pellet was resuspended with SDS sample buffer and heated for 10 min at 70°C. Sample was then loaded onto SDS-PAGE gel and analyzed by immunoblot.

Results

The M3 muscarinic receptor, not the M2 muscarinic receptor, mediates carbachol-induced AKT and MLC phosphorylation. The M2 and M3 muscarinic receptor subtypes are expressed in HASMCs[131]. In order to determine the receptor(s) contributing to carbachol-mediated activation of PI3K/ROCK axis, we examined the effects of carbachol stimulation (10 μM, 10 min) on AKT (S473) and MLC (S19) phosphorylation in primary HASMCs 72 h after transfection with M2R, M3R, or scrambled siRNA. M3R siRNA knockdown reduced M3R protein expression (80 ± 7%)(Figure 1A). Scrambled siRNA had little effect on any of the proteins examined. M3R siRNA attenuated carbachol-induced phosphorylation of AKT (2.55 ± 0.91 fold vs. 0.63 ± 0.49 fold) and phosphorylation of MLC (1.44 ± 0.08 fold vs. 0.261 ± 0.287 fold) compared to scrambled siRNA (Figure 1 B–C). Surprisingly, M2R siRNA induced AKT

phosphorylation (2.5 ± 0.357 fold) in the absence of agonist. Since the M2R couples predominantly to the G protein $G\alpha_i$, we used pertussis toxin (18 h, 1 μg ml^{-1}) to ADP-ribosylate $G\alpha_i$, rendering it inactive, and measured AKT phosphorylation in response to carbachol stimulation (10 μM, 10 min). Incubation with pertussis toxin before carbachol stimulation had little effect when compared to vehicle (0.01% DMSO) (Figure 1D). Pertussis toxin, however, rescued carbachol-induced attenuation of isoproterenol-mediated cAMP elevation (306 ± 23 fold without PTX vs. 461.93 ± 37 fold with PTX).

$G\alpha_{12}$ couples to the M3R in HASMCs. Previous reports in HEK293 cells using GTP photolabelling and $G\alpha_{12}$-specific RGS overexpression demonstrate that $G\alpha_{12}$-is coupled to the M3R[139,142]. To determine whether coupling occurs in HASMCs we used co-immunoprecipitation techniques to pull down the M3R and $G\alpha_{12}$ proteins. These samples were subsequently immunoblotted for the indicated proteins (Figure 2A). When the M3R was co-immunoprecipitated and subsequently probed with $G\alpha_{12}$ antibody, a strong band was present for $G\alpha_{12}$. Upon carbachol stimulation (10 μM, 1 min), the band density decreased. Interestingly, when $G\alpha_{12}$ was immunoprecipitated and subsequently immunoblotted using the M3R antibody, a strong band was also present. Again, band density was decreased upon carbachol stimulation. To further evaluate M3R-$G\alpha_{12}$ coupling, hTERT-immortalized HASMCs overexpressing a GFP-tagged RGS domain of the p115RhoGEF enzyme (p115RhoGEF-RGS-GFP) and corresponding control cell lines were infected with an SRE-luciferase reporter construct that induces luciferase expression upon $G\alpha_{12}$ activation (Figure 2B). These cells were stimulated with carbachol (10 μM, 6 h), lysed, and assayed for luciferase induction using luminescence. Carbachol stimulation elevated luciferase expression (76 ± 18% fold).

Carbachol-induced luciferase induction was attenuated to basal levels in HASMCs expressing p115RhoGEF-RGS (76 ± 18 fold vs. 1.03 ± 0.3 fold with PTX).

$G\alpha_{12}$ mediates M3R-induced activation of PI3K/ROCK axis activation. To determine the contribution of $G\alpha_{12}$ proteins to carbachol-induced PI3K/ROCK axis activation, we used siRNA to knockdown $G\alpha_{12}$ proteins and measured the effects on carbachol-induced phosphorylation of AKT, MYPT1, and MLC. To determine the contribution of $G\alpha_{12}$ proteins to carbachol-mediated activation of PI3K/ROCK axis, we next examined the effects of carbachol stimulation (10 μM, 10 min) on AKT (S473), MYPT1 (T696), and MLC (S19) phosphorylation in primary HASMCs 72 h after transfection with $G\alpha_{12}$ proteins or scrambled siRNA. $G\alpha_{12}$ siRNA knockdown reduced $G\alpha_{12}$ protein expression (73 ± 9%)(Figure 3A) and scrambled siRNA had little effect on any of the proteins examined. $G\alpha_{12}$ siRNA markedly attenuated carbachol-induced phosphorylation of AKT (2.55 ± 0.91 fold vs 1.05 ± 0.54 fold), phosphorylation of MYPT1 (2.1 ± 1.01 fold vs 0.33 ± 0.13 fold), and phosphorylation of MLC (1.44 ± 0.08 fold vs 0.50 ± 0.39 fold) compared to scrambled siRNA (Figure 3B). To complement the $G\alpha_{12}$ siRNA studies, we compared carbachol-induced AKT phosphorylation in hTERT-immortalized HASMC that do/do not express p115RhoGEF-RGS. In p115RhoGEF-RGS-expressing HASMCs, carbachol-induced AKT phosphorylation was attenuated compared to control cell lines (2 ± 0.52 fold vs. 4.47 ± 1.5 fold) (Figure 3C). p115RhoGEF-RGS expression had little effect on $G\alpha_q$ activation, measured by intracellular calcium mobilization (Figure 3D).

$G\alpha_{12}$-mediated activation of PI3K is RhoA-dependent. In order to determine whether $G\alpha_{12}$-mediated activation of PI3K involved Rho and Rac small GTPases as signaling intermediates, we examined the effects of carbachol stimulation (10 μM, 10 min) on AKT (S473) phosphorylation in primary HASMCs 72 h after transfection with RhoA, Rac1 or scrambled siRNA. RhoA and Rac1 siRNA knockdown reduced protein expression (68 ± 19% and 66 ± 16% respectively)(Figure 4A) and scrambled siRNA had little effect on any of the proteins examined. RhoA siRNA attenuated carbachol-induced phosphorylation of AKT (1.98 ± 0.44 fold vs. 0.71 ± 0.13 fold) compared to scrambled siRNA (Figure 4B). To complement siRNA studies, we next used rhosin to inhibit RhoGEFs that activate RhoA and measured AKT phosphorylation in response to carbachol stimulation. Incubation with Rhosin attenuated carbachol-induced phosphorylation of AKT (4.4 ± 0.78 fold vs. 0.65 ± 0.09 fold) compared to vehicle (Figure 4C).

RhoA inhibition promotes bronchodilation of hPCLS. To determine if inhibition of RhoA reverses agonist-induced bronchoconstriction in human small airways (hPCLS). hPCLS were stimulated with carbachol to induce luminal narrowing and subsequently treated with increasing doses of Rhosin or Formoterol to evaluate airway dilation (Figure 5). Rhosin and formoterol reversed carbachol-induced bronchoconstriction with an Emax of 80 ± 5% respectively, as well 100 ± 3% and $\log EC_{50}$ of $1.65e^{21}$ and $4.92e^{7}$.

Discussion

Our study demonstrates a previously unidentified role for $G\alpha_{12}$ in modulating M3R-mediated activation of the PI3K/ROCK axis in HASMCs. We also demonstrate that

$G\alpha_{12}$–mediated activation of PI3K/ROCK axis is RhoA-dependent. Furthermore, we show that inhibition of RhoA blunts carbachol-induced PI3K activation and promotes bronchodilation of human small airways, implicating RhoA as a pivotal mediator of airway tone.

To expand on our previous studies demonstrating that PI3K inhibitors promote bronchodilation of human small airways, we sought to delineate upstream signaling pathways that mediate PI3K activation. We used siRNA and pharmacological tools, as well as HASMCs overexpressing p115RhoGEF-RGS proteins that limit M3R-mediated activation of $G\alpha_{12}$ in order to determine the role in modulating PI3K/ROCK axis activation and HASMC contraction. Our data showed that knockdown of the M3R attenuated carbachol-induced activation of AKT, MYPT1, and MLC phosphorylation. We also showed that $G\alpha_{12}$ coimmunoprecipitated with the M3R, and that p115RhoGEF-RGS expression limited carbachol-mediated induction of SRE-luciferase reporter. $G\alpha_{12}$ siRNA attenuated carbachol-induced activation of AKT, MYPT1, and MLC phosphorylation, and that p115RhoGEF-RGS overexpression limited carbachol-induced activation of AKT. Furthermore, we demonstrated that siRNA and pharmacological inhibition of RhoA blunted carbachol-mediated activation of PI3K, and that RhoA inhibitors induced dilation of hPCLS, implicating RhoA as a pivotal mediator of airway tone.

Despite its lower expression levels, investigators suggest that the $G\alpha_q$-coupled M3 muscarinic receptor, and not the $G\alpha_i$–coupled M2 muscarinic receptor, is the primary subtype responsible for bronchial and tracheal smooth muscle contraction[148–152]. Nonetheless, some studies suggest a role for the M2R in mediating airway smooth muscle contraction in the peripheral airways[153,154]. Our findings using siRNA against the M2R and M3R siRNA, as well as pertussis toxin to inactivate M2R-coupled $G\alpha_i$

demonstrate that the M3R is the dominant receptor mediating the activation of the PI3K/ROCK axis (Figure 1). Incubation with M2R siRNA surprisingly resulted in a robust activation of PI3K that possibly could be related to compensatory expression of proteins that activate PI3K[148]. Our data stand in contrast with studies conducted in rabbit intestinal smooth muscle, where the M2R through Gβγ-dependent signaling, activates PI3K[148]. Interestingly, these cells expressed p110γ isoform of PI3K, that is not expressed in the HASMCs used in our studies[43,46,52,112]. Gβγ proteins are typically thought to signal to the p110γ isoforms of PI3K, not the p110α, p110β, or p110δ isoforms expressed in our HASMCs[155]. This illustrates an important concept that the identical receptors mediate signaling that is tissue and species specific.

GPCR-mediated activation of PI3K can occur through epidermal growth factor receptor (EGFR) transactivation[156]. Previous studies, however, have demonstrated a lack of EGFR phosphorylation induced by carbachol in HASMCs[157]. Additionally, our data demonstrated that EGFR inhibition by tyrphostin had little effect on carbachol-mediated AKT phosphorylation, suggesting that EGFR transactivation mediated by the M3R was unlikely (Supplemental Figure 1). Our next goal was to determine which G-proteins mediate M3R-induced activation of PI3K. Since Gβγ proteins were unlikely to mediate these signals due to the lack of p110γ expression, we tested whether $G\alpha_q$ mediated these signals using a pharmacologic inhibitor FR900359 (UBO-QIC) shown by us and others to specifically inhibit $G\alpha_q$ and not other G-proteins or arrestins[158,159]. These results demonstrated that M3R-induced activation of PI3K was $G\alpha_q$–independent (Supplemental Figure 2).

Since $G\alpha_{12/13}$ family proteins were shown to modulate RhoA/ROCK pathways, we used co-immunoprecipitation and SRE-luciferase reporter expressing HASMCs to

demonstrate whether M3R coupled to $G\alpha_{12}$ in HASMCs (Figure 2). Our results suggest that the M3R indeed is coupled to $G\alpha_{12}$ in HASMCs and that M3R-induced activation of $G\alpha_{12}$ is attenuated by overexpression of the p115RhoGEF-RGS domain. Furthermore, $G\alpha_{12}$ siRNA attenuated carbachol-induced AKT, MYPT1, and MLC phosphorylation, suggesting for the first time that $G\alpha_{12}$ regulates PI3K/ROCK axis activation and MLC phosphorylation in HASMCs. These data agrees with previous studies demonstrating M3R-$G\alpha_{12}$ coupling in HEK293 cells[142]. Other studies have shown a lack of M3R-$G\alpha_{12}$ coupling in murine airway smooth muscle; however, this may be a result of species differences between mice and humans. Our data highlight the importance and necessity of $G\alpha_{12}$ proteins in maintaining HASMC tone through pathways involving the PI3K-ROCK axis.

In order to determine whether $G\alpha_{12}$-mediated activation of the PI3K-ROCK axis involved RhoA, the canonical effector of $G\alpha_{12/13}$ signaling pathways, we used RhoA siRNA and Rhosin, a novel rationally design inhibitor of RhoA, to test whether limiting RhoA signaling would attenuate PI3K activation. Our data show that RhoA siRNA and inhibitors attenuated carbachol-induced AKT phosphorylation, suggesting PI3K as a novel intermediate in $G\alpha_{12}$ signaling. One study has suggested that Rho family GTPases and PI3K function cooperatively, offering an explanation for their relationship in $G\alpha_{12}$ signaling[160]. Further studies are warranted to study cooperatively in HASMCs. A limitation of our data is that the moderate potency of Rhosin allows the potential for off target effects at higher required concentrations. Our RhoA siRNA data, however, support the idea that $G\alpha_{12}$-mediated activation of PI3K is RhoA-dependent.

We demonstrated using hPCLS that RhoA inhibition by rhosin induced bronchodilation comparable to formoterol, an industry standard bronchodilator,

suggesting that inhibition of our newly established $G\alpha_{12}$-mediated signaling pathway provides a novel therapeutic strategy for bronchodilation in asthma.

Our current study and other studies show multiple signaling pathways, kinases, and scaffold proteins regulating ROCK activation in HASMCs including those mediated by arrestins, Src-family kinases, $G\alpha_{12}$, and PI3K[43,161–163]. The multiple intermediates required for ROCK activation suggest the existence of signaling complexes driving ROCK activation. These complexes are composed of multiple kinases and scaffold proteins, and warrant further investigation for several reasons. Their complexity and arrangement is highly cell and tissue specific. Targeting individual signaling intermediates such as kinases has limitations since they are often ubiquitously expressed, allowing for toxicities and side effects, even with inhaled delivery systems. Targeting signaling complex formation would provide greater tissue specificity for therapeutics. Furthermore, using combinations of therapeutics to target constituents of ROCK signaling complexes allows the potential for combination therapies with lower doses of each individual drug. This would allow improved efficacy with fewer side effects, and could provide for improved bronchodilators.

Our data demonstrate a novel coupling of the M3R to $G\alpha_{12}$ HASMCs and that $G\alpha_{12}$ plays a crucial role in contraction through RhoA-dependent activation of the PI3K/ROCK axis. We show that inhibition of RhoA induces bronchodilation in hPCLS, and that targeting $G\alpha_{12}$ signaling may elucidate novel therapeutic targets in asthma.

Figure Legends

Figure 1. Effects of M2R siRNA, M3R siRNA, and pertussis toxin on carbachol-induced AKT and MLC phosphorylation in primary HASMCs. (A-C) Measurement of phosphorylation responses to carbachol (10 μM, 10 min) and protein expression in primary HASMCs after transfection with M2R, M3R, or scrambled siRNA (50 nM, 72 h post-transfection). (A) Effect of scrambled, M2R and M3R siRNA on protein expression. Data normalized to GAPDH expression in the same samples. (B) Effect of carbachol on AKT phosphorylation at S473 (pAKT) after transfection with scrambled, M2R, or M3R siRNA. pAKT data were normalized to total AKT (AKT). (C) Effect of carbachol on MLC phosphorylation at S19 (pMLC) after transfection with scrambled, M2R, or M3R siRNA. pMLC data were normalized to total MLC (MLC). (D) Effect of pertussis toxin (18 h, 1 μg ml^{-1}) on carbachol-induced AKT phosphorylation. Data normalized to Tubulin expression in the same samples. (E) Effect of pertussis toxin (18 h, 1 μg ml^{-1}) on carbachol-induced attenuation of isoproterenol-mediated cAMP production. Data are expressed as fold change over untreated (basal) samples that were measured on the same gel or plate. Data are representative of five independent experiments (n = 5, mean ± SD); statistical comparisons analyzed by unpaired t-test are denoted by lines between tested conditions *P < 0.05.

Figure 2. $G\alpha_{12}$ and M3R coupling in HASMCs. Evaluation of M3R-$G\alpha_{12}$ coupling using co-immunoprecipitation in primary HASMCs and SRE-luciferase reporter in hTERT-immortalized HASMCs expressing p115RhoGEF-RGS. (A) HASMCs were stimulated with carbachol (10 μM, 1 min) and lysates were immunoprecipitated with anti-M3R or anti-$G\alpha_{12}$ antibody and then probed as indicated. Immunoblot is

representative of five independent experiments. (B) hTERT-immortalized HASMCs expressing p115RhoGEF-RGS and control hTERT-immortalized HASMCs were infected with SRE-luciferase reporter. After carbachol stimulation (10 μM, 6 h), cells were lysed and SRE-luciferase reporter activity was measured. Data are expressed as fold change over untreated (basal) samples that were measured on the same plate. Data are representative of six independent experiments (n = 6, mean ± SD); statistical comparisons analyzed by unpaired t-test are denoted by lines between tested conditions $^{*}P < 0.05$.

Figure 3. Effects of $G\alpha_{12}$ siRNA and p115RhoGEF-RGS overexpression on M3R-mediated activation of the PI3K/ROCK/MLC axis in HASMCs. (A-C) Measurement of phosphorylation responses to carbachol (10 μM, 10 min) and protein expression in primary HASMCs after transfection with $G\alpha_{12}$ or scrambled siRNA (50 nM, 72 h post-transfection). (A) Effect of $G\alpha_{12}$ or scrambled siRNA on protein expression. Data normalized to Tubulin expression in the same samples. (B) Effect of carbachol on AKT, MYPT1, and MLC phosphorylation at S473 (pAKT), T696 (pMYPT1), and S19 (pMLC) after transfection with $G\alpha_{12}$ or scrambled siRNA. pAKT, pMYPT1, and pMLC data were normalized to total AKT (AKT), total MYPT1 (MYPT1), and total MLC (MLC). (C) Effect of p115RhoGEF-RGS overexpression on carbachol-induced AKT phosphorylation in hTERT-immortalized HASMCs. (D) Effect of p115RhoGEF-RGS overexpression on carbachol-induced intracellular calcium mobilization in hTERT-immortalized HASMCs. Data are expressed as fold change over untreated (basal) samples that were measured on the same gel or plate. Data are representative of five

independent experiments (n = 5, mean ± SD); statistical comparisons analyzed by unpaired t-test are denoted by lines between tested conditions *P < 0.05.

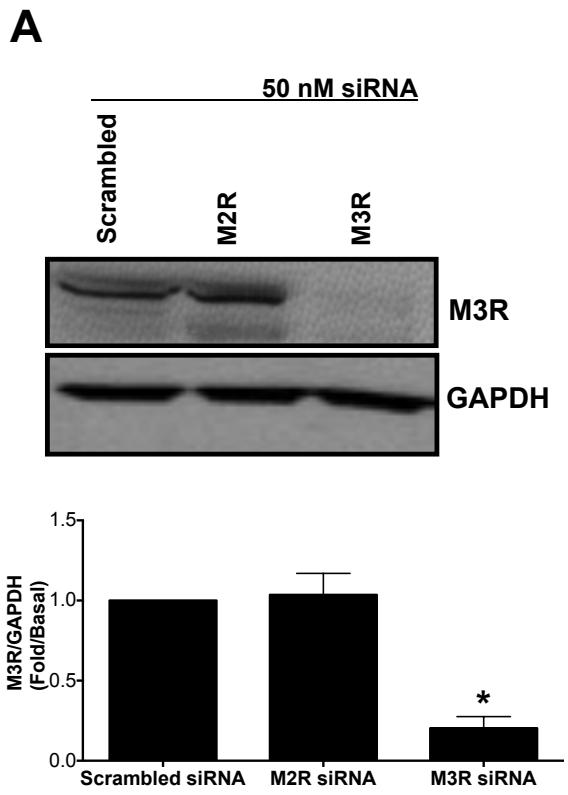
Figure 4. Effects of RhoA inhibitors and siRNA on M3R-mediated activation of PI3K in primary HASMCs. Measurement of phosphorylation responses to carbachol (10 μM, 10 min) and protein expression in primary HASMCs after transfection with RhoA, Rac1, or scrambled siRNA (50 nM, 72 h post-transfection) or after incubation with Rhosin (RhoA inhibitor) (10 μM, 30 min). (A) Effect of rhosin on carbachol-induced AKT phosphorylation at S473 (pAKT). (B) Effect of scrambled, RhoA and Rac1 siRNA on protein expression. Data normalized to MLC expression in the same samples. (C) Effect of carbachol on AKT phosphorylation at S473 (pAKT) after transfection with RhoA, Rac1, or scrambled siRNA. pAKT data were normalized to total AKT (AKT). Data are expressed as fold change over untreated (basal) samples that were measured on the same gel. Data are representative of five independent experiments (n = 5, mean ± SD); statistical comparisons analyzed by unpaired t-test are denoted by lines between tested conditions *P < 0.05.

Figure 5. Effects of RhoA inhibitors on bronchodilation of hPCLS. Measurement of bronchodilation responses to rhosin in hPCLS. Airways were preconstricted to carbachol (10^{-8}–10^{-4} M) prior to dilation to rhosin or formoterol (10^{-10}–10^{-4} M). Data were normalized to maximum forskolin response (10 μM) that was given after max dose of formoterol or rhosin. (Maximal airway dilation (Emax, ANOVA, P = 0.03) and AUC (P = 0.004) for rhosin was significantly different than

formoterol-induced dilation. Data are representative of *6* slices per condition, with bars representing mean ± SD.

Figures

Figure 1



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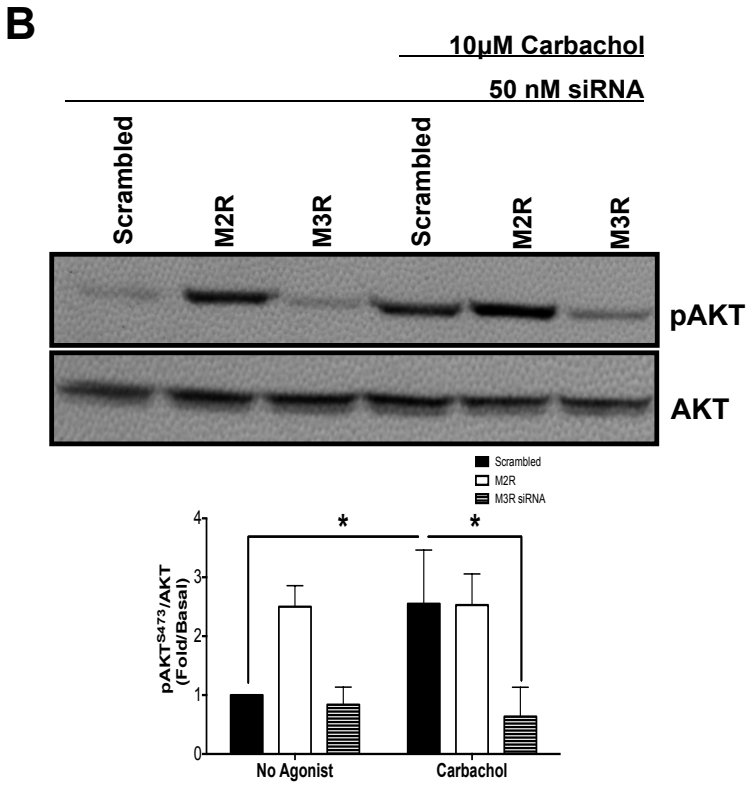
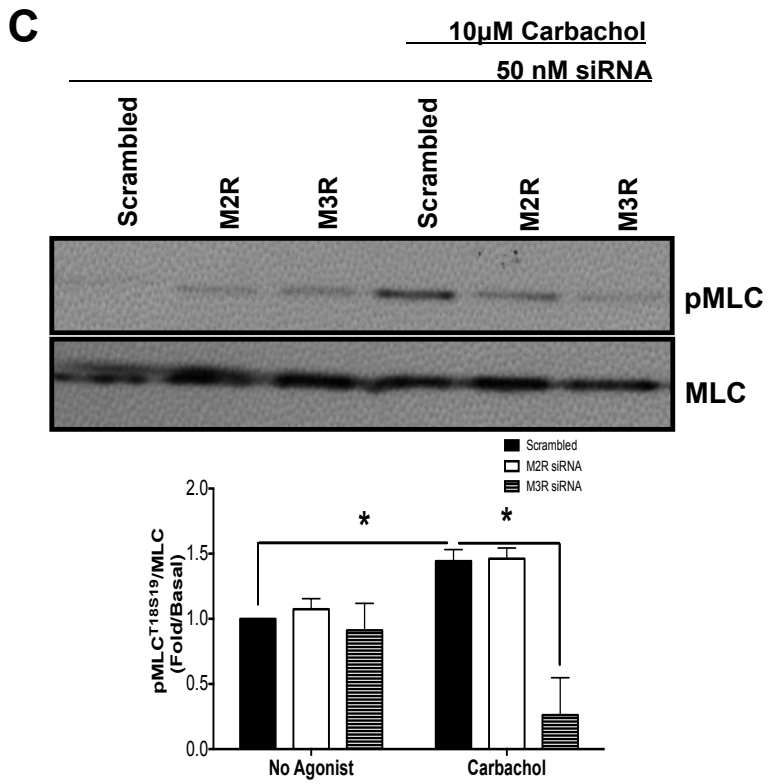


Figure 1 (cont.)



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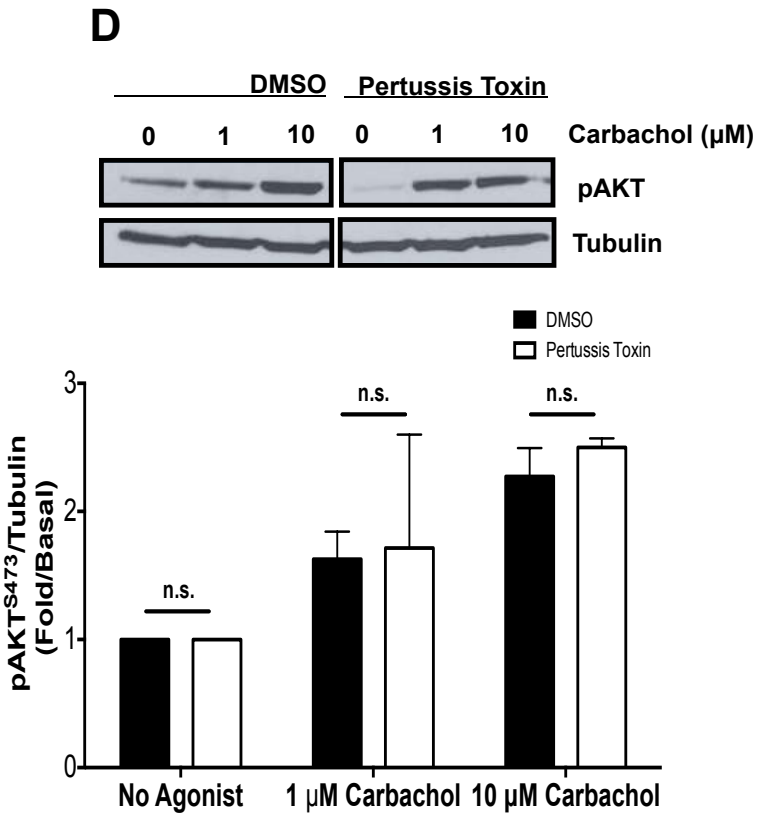


Figure 1 (cont.)

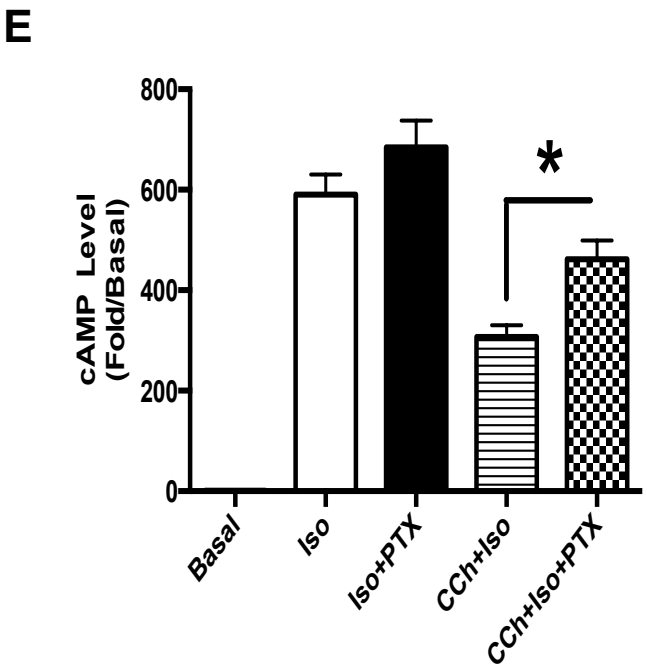


Figure 2

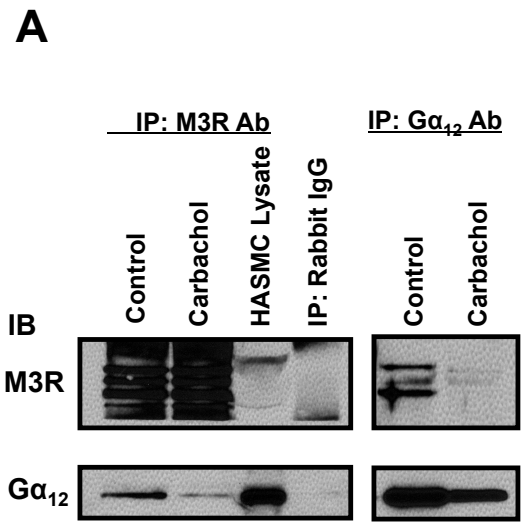

A
IP: M3R Ab
IP: Gα12 Ab
Control
Carbachol
HASMC Lysate
IP: Rabbit IgG
Control
Carbachol
IB
M3R
Gα12

Figure 2 (cont.)

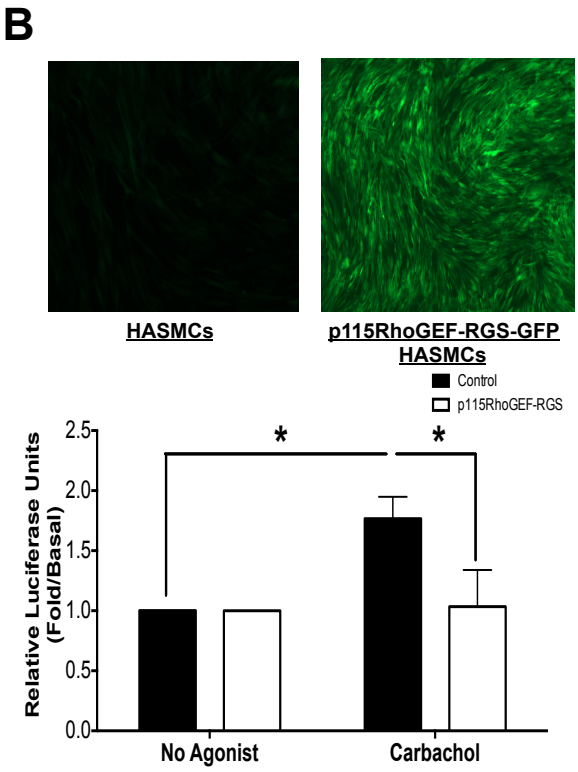


Figure 3

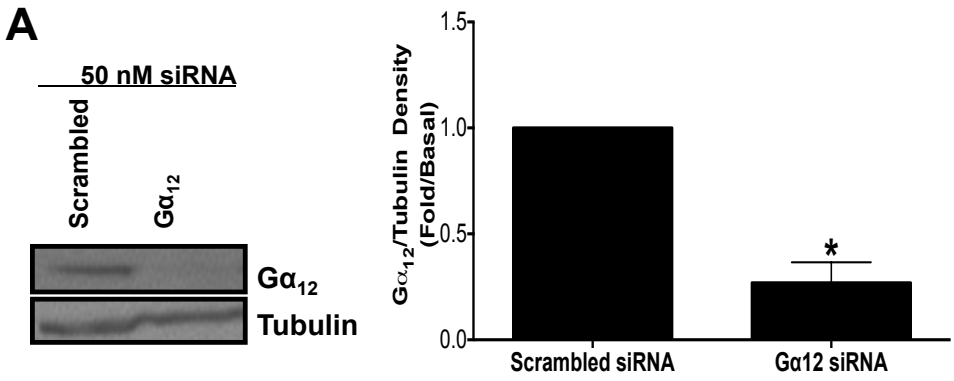

A
50 nM siRNA
Scrambled
Gα12
Gα12
Tubulin
Gα12/Tubulin Density (Fold/Basal)
1.5
1.0
0.5
0.0
*
Scrambled siRNA
Gα12 siRNA

Figure 3 (cont.)

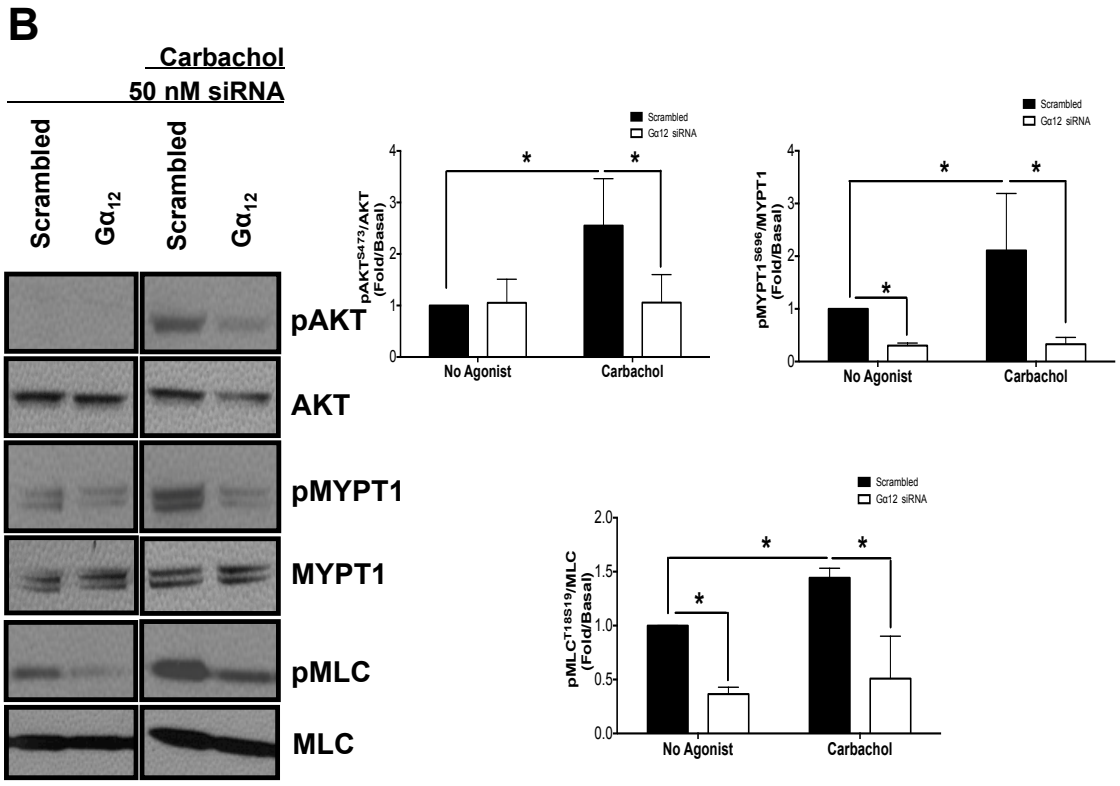


Figure 3 (cont.)

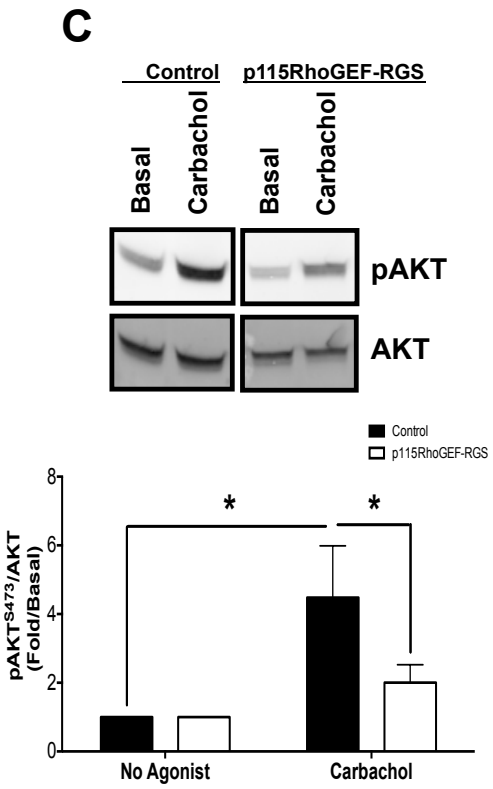


Figure 3 (cont.)

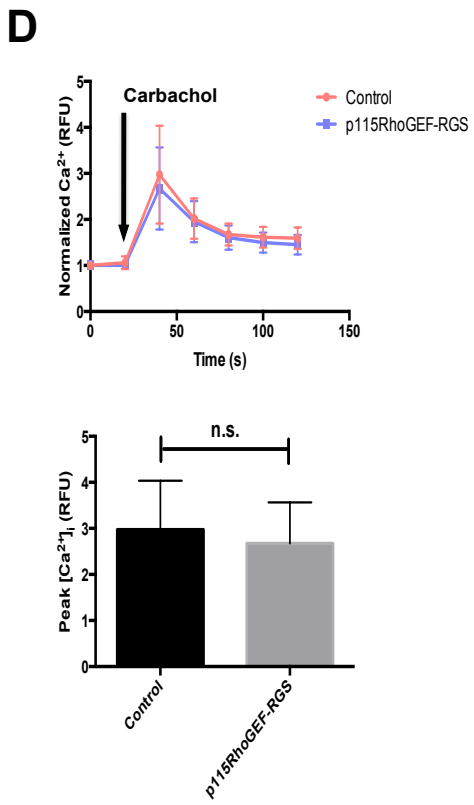


Figure 4

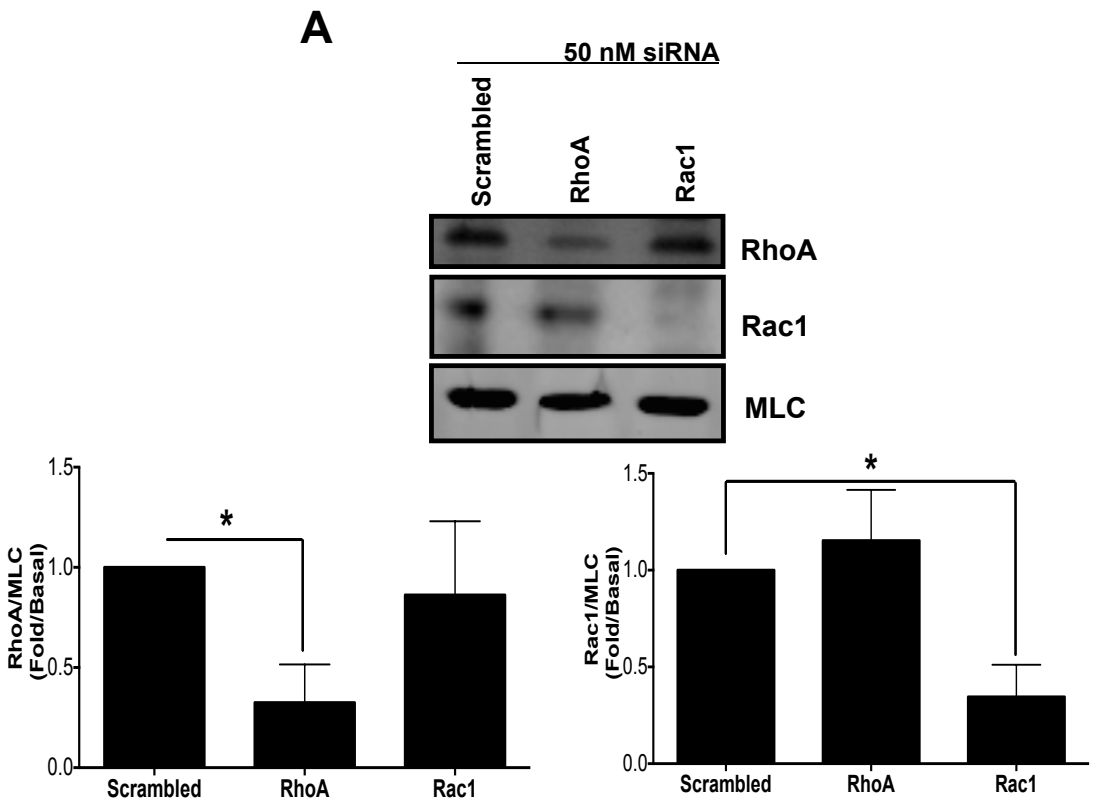

A
50 nM siRNA
Scrambled
RhoA
Rac1
RhoA
Rac1
MLC
RhoA/MLC (Fold/Basal)
1.5
1.0
0.5
0.0
*
Scrambled
RhoA
Rac1
Rac1/MLC (Fold/Basal)
1.5
1.0
0.5
0.0
*
Scrambled
RhoA
Rac1

Figure 4 (cont.)

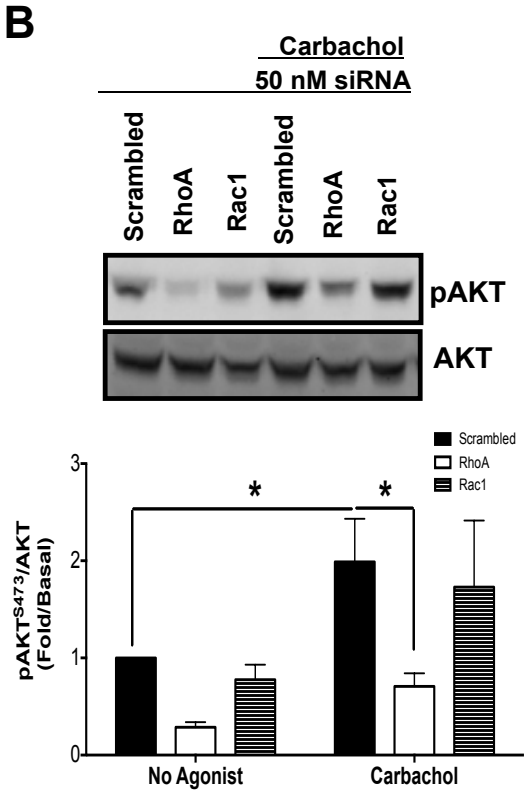


Figure 4 (cont.)

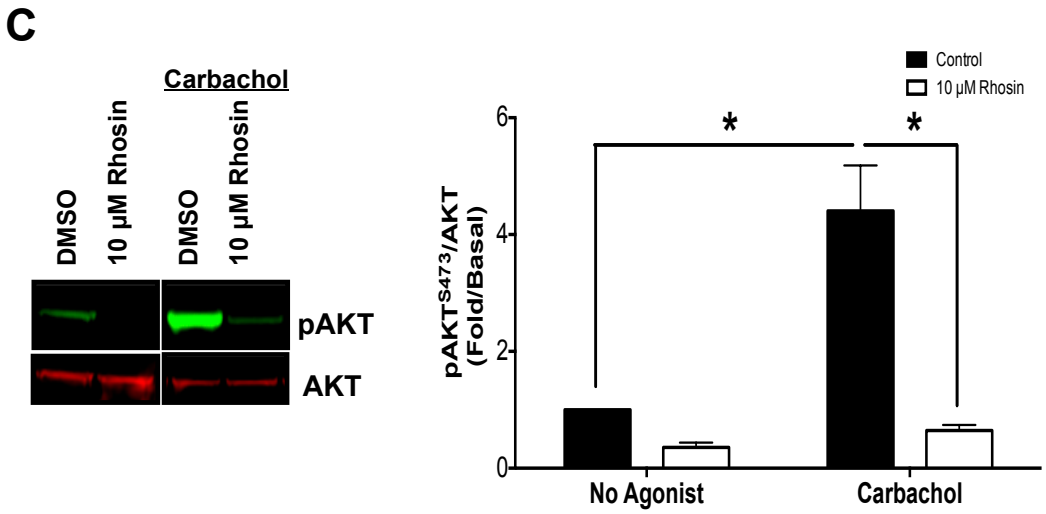
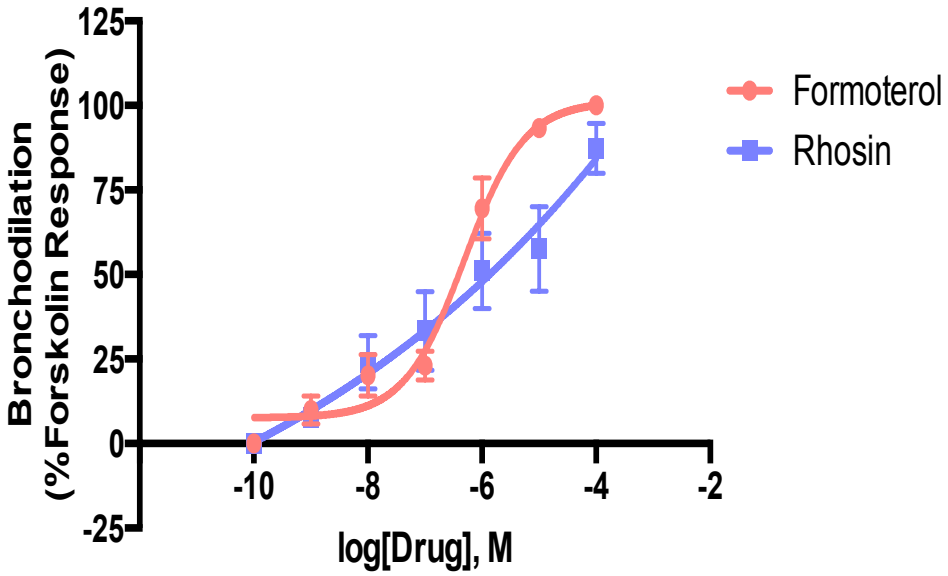


Figure 5


Bronchodilation
(%Forskolin Response)
125
100
75
50
25
0
-25
-10
-8
-6
-4
-2
log[Drug], M
Formoterol
Rhosin

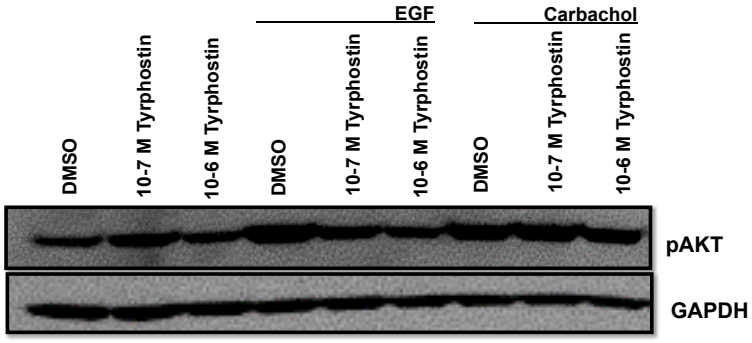
Supplemental Figure Legends

Supplemental Figure 1. Effect of EGFR inhibition on carbachol-induced AKT phosphorylation. EGFR inhibition by tyrphostin (1 μM) inhibited EGF-induced (1 ng/ml) AKT phosphorylation but had little effect on carbachol-induced (10 μM), AKT phosphorylation in HASMC.

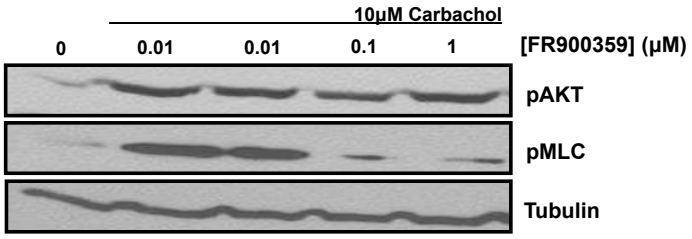
Supplemental Figure 2. Effect of $G\alpha_q$ inhibition on carbachol-induced AKT phosphorylation. $G\alpha_q$ inhibition by FR900359 has little effect on carbachol-induced (10 μM), AKT phosphorylation in HASMC.

Supplemental Figures

Supplemental Figure 1



Supplemental Figure 2



CHAPTER 4: Discussion

Edwin J. Yoo[1]

[1]Rutgers Institute for Translational Medicine and Science, Child Health Institute , Rutgers University, New Brunswick, NJ, USA,

Phosphoinositide 3-kinase inhibitors: novel bronchodilators

Asthma management has historically focused on: 1) using bronchodilators that bind G protein-coupled receptors (GPCRs) to directly inhibit airway smooth muscle (ASM) shortening, prevent (bronchoprotect) or reverse bronchoconstriction (bronchodilate); and 2) controlling airway inflammation to limit mediators that induce ASM contraction or other structural changes that impede airflow (mucus secretion, airway edema). β-2 agonists, the industry standard bronchodilator, bind to the β-2 adrenergic receptor on airway smooth muscle (ASM) and inducing $G\alpha_s$-mediated cAMP elevation and protein kinase A (PKA) activation. PKA will then limit signaling pathways that induce ASM shortening such as intracellular calcium mobilization, rho kinase (ROCK) activation, and myosin light chain kinase (MLCK) activation. Unfortunately, about 55% of asthmatics have suboptimal control of their symptoms[164].

Despite β-2 agonists' ability to prevent and mitigate asthma exacerbations, they have limitations especially concerning efficacy and safety. Studies have shown that β-2 agonist use can result in adverse patient outcomes, β-2 adrenergic receptor tachyphylaxis, deterioration of asthma control, and death[165,6,5]. New therapeutics are needed to overcome these limitations. New formulations of β-2 agonists offer improved specificity and duration of action. Unfortunately, truly novel therapeutics that overcome the limitations of existing drugs have yet to emerge.

Basic science has identified several novel targets for bronchodilation but none of these strategies have translated successfully into clinical utility. Receptor antagonists that block bronchoconstricting agonists include antagonists to the muscarinic, histamine, and leukotriene receptors to prevent shortening of ASM[135]. Due to the multitude of bronchoconstricting mediators present in asthma pathology, however, none

have been uniformly effective. Several alternative $G\alpha_s$-coupled receptors have been discovered including bitter taste receptors, the pH sensing receptor OGR1, and prostaglandin-binding EP receptors[135]. Regrettably, these receptors lack potent and specific ligands, and have similar limitations regarding receptor tachphylaxis.

ROCK inhibitors have generated much excitement in the field[81]. Since ROCK activation is necessary to maintain ASM tone by inhibiting MLC phosphatase, ROCK inhibitors allow the constitutively active MLC phosphatase to de-phosphorylate MLC and allow relaxation of ASM. ROCK inhibitors blunt ASM contraction and induce bronchodilation *in vitro*, *ex vivo, and in vivo*[166,167,106,168–170]. The limitation with targeting ROCK is that it is ubiquitously expressed, making the potential for undesired side effects high. Studies have shown that ROCK can induce hypotension as a result of vasodilation[171]. Despite the plethora of promising findings from animal studies that demonstrate effective bronchodilation, clinical trials in humans have not been published. Inhibiting ROCK is an effective approach to induce bronchodilation, but strategies need to be developed to avoid off target effects. These strategies can include improved targeting to airway smooth muscle, or elucidating the signaling pathways that drive ROCK activation in order to identify novel mediators that are not expressed in all smooth muscle cells.

We present a novel strategy to induce bronchodilation in human airways by inhibiting the p110δ isoform of phosphoinositide 3-kinase (PI3Kδ)[43]. In primary human ASM cells (HASMCs), we demonstrated that PI3Kδ modulates ROCK activation, and that inhibition of PI3Kδ using a FDA approved drug Cal101 (Idelalisib) attenuates ROCK activation. Cal101 induced relaxation of cultured HASMCs by inhibiting PI3Kδ-mediated activation of ROCK. Using human precision-cut lung slices, we also demonstrated that Cal101 induces bronchodilation of human small airways as effectively as formoterol, an

industry standard β-2 agonist. Cal101 displayed similar potency to Y27632, a ROCK inhibitor. In the context of β-2 agonist tachyphylaxis, the effectiveness of β-2 agonists were significantly attenuated while the effectiveness of Cal101 was not. Thus, our results suggest that PI3Kδ inhibitors represent a novel class of bronchodilators.

Advantage and limitations of PI3Kδ inhibitors as asthma therapeutics

Inhibition of PI3Kδ is an exciting strategy because it can potentially overcome limitations of current therapies. PI3Kδ inhibitors are small molecules that can bypass cell surface receptors and bind to their intracellular target. By bypassing receptors, PI3Kδ inhibitors overcomes tachyphylaxis due to receptor desensitization and internalization. Furthermore, PI3Kδ inhibitors can reverse β-2 receptor desensitization and tachyphylaxis to a degree[172]. Since PI3Kδ inhibitors can reverse airways pre-constricted to multiple agonists, these inhibitors potentially can be effective in the presence of the multiple mediators present in the airway during asthma exacerbations, unlike cell surface receptor antagonists. PI3Kδ exhibits tissue-selective expression and is not expressed ubiquitously like PI3Kα/PI3Kβ isoforms and ROCK[173] (Table 1.1). Our studies provide a rationale for PI3Kδ inhibitors to have the potential for a better therapeutic window, especially with inhaled delivery systems.

In the context of asthma, PI3Kδ inhibitors have been extensively studied due to their anti-inflammatory properties[173]. Studies showed these inhibitors are effective anti-inflammatory agents despite corticosteroid insensitivity. As a result of many successful studies, PI3Kδ inhibitors have been moving through clinical trials for asthma steadily. GSK2269557, RV1729, and IPI-145 have progressed into Phase 2 clinical trials as inhaled formulations (Table 2.1). Furthermore, Idelalisib was FDA approved in 2014 for indolent non-Hodgkin's lymphoma[83]. The success of PI3Kδ inhibitors in clinical trials

makes the potential for PI3Kδ inhibitors to translate into the clinic as bronchodilators more likely than other potential candidates. The capability to function as a bronchodilator and as an anti-inflammatory provides evidence to suggest that PI3Kδ inhibitors may complement β-2 agonist and corticosteroids in the treatment of asthma.

Like all therapeutic agents, challenges exist with PI3Kδ inhibitors. Kinase inhibitors have limitations including specificity and adverse effects, due in part to off-target effects and loss of efficacy over time. Kinase inhibitors usually target the ATP-binding pocket and since ATP is present in high concentrations, kinase inhibitors typically require nanomolar potency to be effective. Unfortunately, targeting the ATP-binding pocket attenuates specificity and evokes greater potential for off-target effects. Recent studies suggest that kinase inhibitors with high specificity can be developed based on an understanding of the cocrystal structure of the ATP-ligand binding site of kinases[174]. Additionally, approaches targeting allosteric sites that are distant from the ATP-binding sites have been successful with over 10 allosteric kinase inhibitors now in clinical development[175]. Further, we can mitigate off-target effects for airway disease with topical delivery systems such as inhalers. These systems deliver drugs directly into the airway, limiting systemic side effects.

Molecular mechanisms of GPCR-mediated PI3K and ROCK activation

GPCR-mediated regulation of ROCK is complex. Upstream elements are recruited to activate ROCK in HASMC. Some examples include G proteins, arrestins, and src family kinases. Elucidating these upstream elements become especially important in airway biology because disruption of their action serves as potential therapeutic strategies. To delineate the upstream signaling pathways involved in the regulation of PI3K and ROCK, we systematically tested the signaling elements proximal

to the pro-contractile muscarinic acetylcholine receptor 3 (M3R) that mediate activation of PI3K and ROCK. We used siRNA and pharmacologic inhibitors in hPCLS and primary HASMCs including expression of p115RhoGEF-RGS domain proteins that limit $G\alpha_{12}$ signaling. For the first time, we demonstrate that the M3R does couple to $G\alpha_{12}$ in HASMCs. We have demonstrated that $G\alpha_{12}$ is necessary for the activation of the PI3K/ROCK axis and that activation is dependent on RhoA (Figure 1). Finally, we demonstrate that inhibitors of RhoA can induce bronchodilation of hPCLS.

In airway biology, investigators have focused on understanding how GPCR's mediate ROCK activation. Recently, several signaling molecules have emerged as necessary for GPCR-mediated activation of ROCK including arrestins, src family kinases, focal adhesion kinases, G-proteins, and PI3K[43,162,163,161]. Interestingly, these molecules are necessary but not sufficient for ROCK activation because multiple pathways are required for ROCK activation to occur that include scaffolding proteins, as well as enzymes such as kinases. The assembly of signaling scaffolds poses interesting therapeutic targets.

First, the necessity of multiple kinases for ROCK activation suggests that combinations of kinase inhibitors at lower doses to disrupt the signaling cascade at multiple nodes can effectively blunt downstream activation of ROCK. Inhibiting multiple kinases may increase efficacy since these agents may be additive or act synergistically[81]. Additionally, inhibiting multiple kinases decreases the chances of developing resistance by blocking pathways that are upregulated in compensatory response to mono-kinase inhibition, that occurs with continued use of inhibitors. As an example, several multikinase inhibitors have been developed in inhaled formulations for the treatment of steroid-resistant inflammation in severe asthma and COPD[81]. These drugs inhibit p38α, p38γ, and Hck, and are more effective than p38α inhibitors alone in

suppressing the release of inflammatory cytokines from macrophages in vitro and in treating inflammation in mice exposed to cigarette smoke.

Secondly, the assembly of signaling complexes suggests that we can target protein-protein interactions pharmacologically in order to destabilize complex formation. With kinase inhibitors, specificity and design of pharmacologic agents remains challenging since there exists limited structural features like the ATP-binding that can be targeted pharmacologically. With signaling complexes, there are more structural features that provide targets that offer attractive possibilities for developing improved therapeutics. Since each cell has a unique array of molecules that assemble to activate ROCK, targeting multiple kinases and targeting signaling complexes in ASM would offer greater specificity and less potential adverse effects. Coupled with inhaled delivery systems, this would suggest powerful new strategies to develop improved bronchodilator therapies.

Future Directions

To further investigate signaling cascades and signaling complexes, we can employ sophisticated mass spectrometry-based proteomics strategies to determine quantitative and temporal dynamics of agonist-mediated activation of ROCK[176]. Bioinformatics software can then interpret this information to generate an unbiased list of phosphorylated proteins responding to the stimulus. This information can be further analyzed to determine kinases that are activated upon stimulation. Kinases with available inhibitors or siRNA can then be tested for contribution to ROCK signaling.

To determine complex formation, we can use proteomics again but with a different experimental design. We can express ROCK or scaffolding proteins like arrestins with a C terminal FLAG tag epitope in HASMCs[177]f. Following agonist

stimulation, we can immunoprecipitate these proteins and all interacting proteins using anti-FLAG antibodies. These samples can then undergo proteomic analysis to determine proteins associated with ROCK or scaffolding proteins like arrestins. Protein interactions can then perturbed using siRNA and inhibitors in order to determine their contribution to the formation of ROCK signaling complexes.

To extend our discovery that PI3K inhibitors can bronchodilate, we will conduct hPCLS experiments testing PI3K inhibitors in combination with β-2 agonists to determine if they display additive or synergistic effects. Studies suggest that muscarinic antagonists can act synergistically with β-2 agonists[178]. We will investigate whether PI3K inhibitors and β-2 agonists act synergistically or additively. For this experiment, we can establish dose response curves for each compound and determine isoeffective concentrations. We will then examine dual drug interactions using isoeffective concentrations. Finally we can use statistical models such as the Bliss Independence model to determine whether drugs are additive, synergistic, or antagonistic[178].

Our observations that $G\alpha_{12}$ and $G\alpha_q$ proteins are required to activate RhoA and ROCK were surprising. $G\alpha_{12}$ are predominantly coupled to GPCRs that are also coupled to $G\alpha_q$. $G\alpha_q$ can also activate RhoA and ROCK and begs the question what is the role of $G\alpha_{12}$? Interestingly, $G\alpha_{12}$ manifests orders of magnitude slower GDP on/off rates than other G proteins, suggesting that activation is slower and prolonged[136]. Accordingly, I posit that cells need $G\alpha_q$ for the initial mobilization of calcium and generation of tone and that $G\alpha_{12}$ sustains signaling and maintains bronchomotor tone. This is supported by data showing that Cal101 induces bronchodilation when given after bronchoconstricting to agonist, but fails to prevent bronchoconstriction when given before agonists and suggests that Cal101 has little effect when given before agonist when $G\alpha_q$ is active. After agonist stimulation and attenuation of $G\alpha_q$ activation pathways, however, Cal101 induces

bronchodilation by inhibiting pathways activated by $G\alpha_{12}$[43]. Accordingly, this suggests that $G\alpha_q$ inhibition prevents bronchoconstriction when given before bronchoconstriction, but should be ineffective in inducing bronchodilation when given after agonist since this is when $G\alpha_{12}$ signaling dominates[158]. We well pursue this hypothesis using BRET biosensor assays to determine the temporal aspects of G protein signaling in hPCLS experiments[162].

Conclusions

β-2 agonists are the industry standard bronchodilator for acute rescue therapy during asthma exacerbations, as well as controller therapy to prevent asthma symptoms. Unfortunately, patients with asthma still suffer from suboptimal control due to limitations including safety, efficacy, and tachyphylaxis. Promising candidates have emerged such as ROCK inhibitors. Due to limitations such as unacceptable adverse effects, these inhibitors have unfortunately been unable to progress into clinical trials.

In this dissertation, we determined that inhibitors of PI3Kδ could function as novel bronchodilating agents by blunting signaling that leads to ROCK activation. PI3Kδ inhibitors promote bronchodilation with similar efficacy as β-2 agonists and ROCK inhibitors. PI3Kδ is not ubiquitously expressed like ROCK, and may therefore offer a better therapeutic window. Importantly, we also found that PI3Kδ inhibitors evoke bronchodilation in the context of β-2 adrenergic receptor desensitization and tachyphylaxis, suggesting that PI3Kδ inhibitors may be promising alternate therapeutics when β-2 agonists fail to be effective. In cancer studies, PI3Kδ inhibitors have been FDA approved for non-Hodgkin's lymphoma and others remain in Phase 2 clinical trials for asthma. Success in clinical trials makes PI3Kδ inhibitors exciting candidates to be repurposed for asthma.

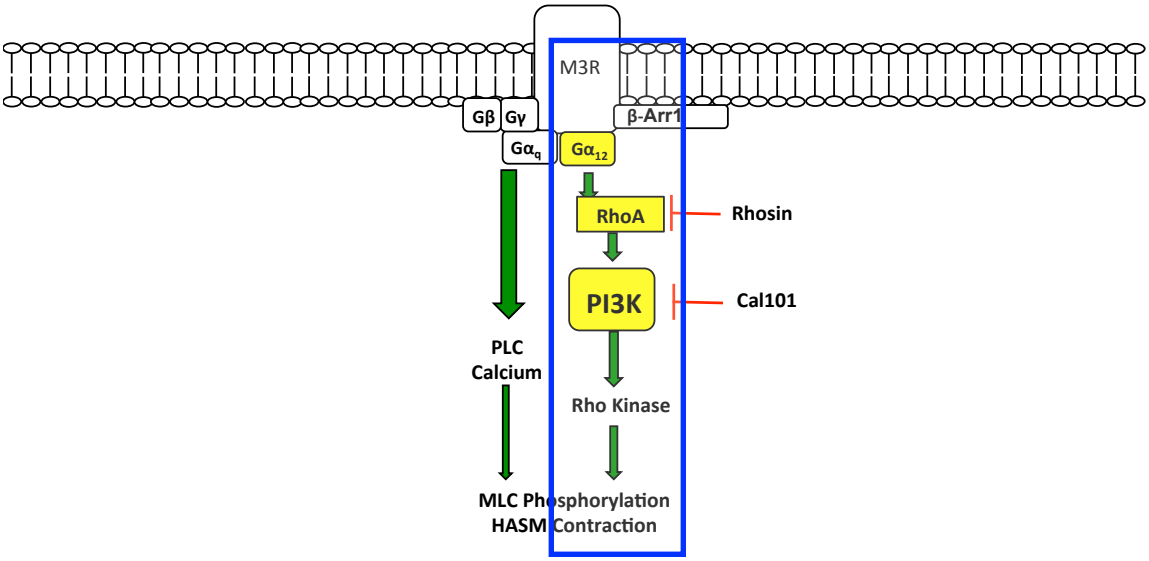
We have also demonstrated for the first time that $G\alpha_{12}$ mediates M3R-induced activation of PI3K and subsequently ROCK. These results reveal a completely novel pathway regulating M3R signaling that is necessary to elicit HASMC shortening. Importantly, our work has uncovered several new targets for bronchodilation and has offered new avenues for translation of science.

Figure Legends

Figure 1. Summary of $G\alpha_{12}$-mediated ROCK Signaling in HASMC. Upon stimulation by acetylcholine, the M3 muscarinic acetylcholine receptor activates $G\alpha_{12}$, resulting in RhoA-mediated activation of PI3K and ROCK.

Figures

Figure 1



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